VU VRIJE UNIVERSITEIT AMSTERDAM

VU Research Portal

Arginine/Nitric Oxide Metabolism in Surgical Oncology

Buijs, N.

2018

document version
Publisher's PDF, also known as Version of record

Link to publication in VU Research Portal

citation for published version (APA)
Buijs, N. (2018). *Arginine/Nitric Oxide Metabolism in Surgical Oncology*. [, Vrije Universiteit Amsterdam].

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal ?

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

E-mail address:
vuresearchportal.ub@vu.nl

Download date: 05. May. 2023

ARGININE/NITRIC OXIDE METABOLISM IN SURGICAL ONCOLOGY

Nikki Buijs

ARGININE/NITRIC OXIDE METABOLISM IN SURGICAL ONCOLOGY

Nikki Buijs

Thesis App:
thesisapps.com/buijs
App provided by the Department of Surgery, VU University Medical Center, Amsterdam

The printing of this thesis was financially supported by:
VU University Medical Center, Egbers Stichting, Nationaal Fonds tegen Kanker, Fresenius Kabi B.V., Nutricia Advanced Medical Nutrition.

Cover and book layout by AgileColor
Printed by Ridderprint BV
ISBN: 978-94-6299-973-2

© Nikki Buijs, 2018. All rights reserved. No part of this thesis may be reproduced, stored in a retrieval system, or transmitted in any form or by any means without prior permission of the author.

VRIJE UNIVERSITEIT

ARGININE/NITRIC OXIDE METABOLISM IN SURGICAL ONCOLOGY

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor
aan de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. V. Subramaniam,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de Faculteit der Geneeskunde
op donderdag 21 juni 2018 om 11.45 uur
in de aula van de universiteit,
De Boelelaan 1105

Door

Nikki Buijs
geboren te Amersfoort

promotor:	prof.dr. P.A.M. van Leeuwen
copromotor:	dr. A.P.J. Houdijk

Contents

PART II

ARGININE/NO METABOLISM IN SURGICAL ONCOLOGY

General introduction

1

Arginine

Arginine is a conditionally essential amino acid, meaning that exogenous and endogenous arginine supply meet the need of the adult human body in physiological conditions, however during growth and pathological conditions *de novo* arginine production becomes inadequate (1). Arginine is a molecule of particular interest because it plays a variety of roles in many different pathways in multiple cell types. Arginine is an important regulator of protein synthesis and proteolysis and it serves as the precursor for nitric oxide (NO), creatine, agmatine, polyamines, proline and glutamate (2). Accordingly, an arginine deficiency has the potential to disturb many cellular and organ functions(3).

Besides being a precursor for several protein metabolites, arginine has important metabolic functions on its own. Arginine is a component of proteins and is an intermediate in the urea cycle in the liver. The urea cycle regulates the body homeostasis by ammonia detoxification and metabolically generated bicarbonate elimination (pH regulation) (4). Also, arginine affects the metabolism of amino acids, protein, glucose and fatty acids on whole-body level by stimulating the expression of several hormones, including pancreatic hormones (glucagon and insulin), anterior pituitary hormones (growth hormone and prolactin) and placental lactogen (5). In addition, arginine is an important regulator of the complex mechanisms initiating protein synthesis and protein breakdown.

Furthermore, arginine on its own is essential for an adequate immune response since it is the substrate for T-lymphocyte proliferation. T-lymphocytes depend on adequate arginine levels for proliferation, the expression of the T-lymphocyte receptor complex and the ζ-chain peptide, and the development of immunological memory (6,7).

Free arginine is derived from the diet, endogenous synthesis, and proteolysis. Approximately 5-6 gram arginine is from daily dietary (exogenous) origin and whole-body arginine turnover ranges between 15-20 grams per day (8,9). Arginine can also be generated from protein breakdown or endogenous synthesis in the *de novo* arginine pathway. Close to 85% of arginine entering the circulation finds its origin in protein turnover. Although contributing for only 10-15% to total arginine production in healthy circumstances, *de novo* arginine synthesis becomes more important for arginine availability in disease states (10). *De novo* arginine production involves the intestinal-renal axis (Figure 1). The ultimate source for endogenous arginine production is glutamine, which is the

most abundant amino acid in the human body, mainly stored in skeletal muscle (11,12). Glutamine is used for the formation of citrulline in enterocytes of the small intestine. Citrulline is scarce in the human diet and citrulline production in the small intestine is responsible for almost the whole-body citrulline content (13). This is illustrated by the fact that arginine becomes an essential amino acid when citrulline formation in the small intestine is inhibited, for example after partial surgical resection of the small intestine (14). Furthermore, low citrulline levels are related to decreased glutamine concentrations as observed in nutritional depletion (15). In physiological conditions, the citrulline turnover in the liver is not significant and therefore the major part of intestinal produced citrulline enters the systemic circulation (16). Endogenous arginine synthesis from citrulline takes place in the proximal tubules of the kidneys by the enzymes argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL). Interestingly, it has been observed that glutamine supplementation increases citrulline and arginine concentrations and may enhance renal citrulline uptake and arginine release (17).

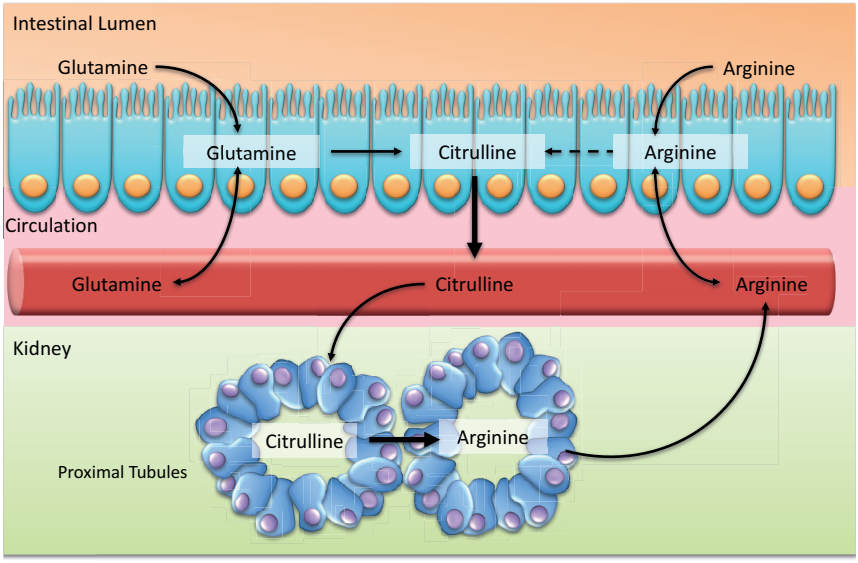

FIGURE 1 | The intestinal-renal axis for arginine *de novo* synthesis.

The first step in arginine metabolism is the transportation across the cellular membrane through cationic amino acid transporters (CAT), which is a sodium dependent mechanism with a substrate specificity, also known as system y^+

(18,19). Once intracellular, arginase and nitric oxide synthase (NOS) are the main arginine catabolizing enzymes. Arginase converts arginine into ornithine and urea and is the enzyme regulating arginine levels throughout the body (20,21). There are two forms of arginase: the hepatic-form catalyzing the final step in the urea cycle, generally referred to as arginase I. The extra-hepatic isoform is arginase II. Apart from the location, these two types differ in several ways from each other. Arginase I is a cytosolic enzyme, while arginase II is a mitochondrial enzyme. Also the expression of the enzymes is regulated in different ways, mostly depending on cell type and the type of stimulus for messenger RNA (21). However, the enzymatic properties of the isoenzymes are similar, and both efficiently convert arginine into ornithine and urea (22).

NOS uses arginine as the sole precursor for NO synthesis. NO plays key roles in multiple regulating mechanisms in various organ systems. The level of NO synthesis is very important in the signaling effect and therefore should be accurately regulated. This is managed by means of one of the three isoforms of NOS: originated in neuronal cells (nNOS), in endothelial cells (eNOS) and an inducible form (iNOS) (3,23). The first two forms are constitutively expressed at low levels in a variety of cell types and synthesize NO in low amounts in a dynamic manner. Conversely, iNOS is normally not expressed in most cells and is highly inducible by inflammatory factors. Once expressed, iNOS is continuously active in generating NO (21,24).

The formation of NO is in part regulated by arginine availability, and arginase is the enzyme able to affect arginine plasma levels (25). For example, low levels of arginine are found in the liver, because of abundant levels of hepatic arginase. Therefore, arginine synthesized in the liver as part of the urea cycle, is not used for hepatic NO synthesis. Another important regulator of NO production are the methylarginines. Arginine residues can be methylated by protein arginine methyltransferases. Asymmetric dimethylarginine (ADMA) is a competitive inhibitor for all NOS isoforms (26). The arginine/ADMA ratio is an indicator for NOS activity and therefore NO production. ADMA is predominantly catalyzed by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). Thus, ADMA and DDAH have an important regulatory role in NO production and the balance between arginine and ADMA (arginine/ADMA ratio) is the preserving factor in this pathway (27).

Nitric oxide

As described above, arginine is the sole precursor for NO formation through the NOS isoenzymes and many of arginine's effects are based on the action of NO. The metabolic role of NO has been studied extensively because of its broad signaling effects in multiple organ systems.

The role of NO is widely studied in the cardiovascular system. There is a physiologic NO-dependent vasodilator tone that is essential for the regulation of organ blood flow and pressure, which is dependent on the arginine/ADMA ratio (27). Endothelial-derived NO inhibits platelet aggregation and platelets themselves generate NO as a negative feed-back mechanism, to inhibit their activation. eNOS maintains endothelial function and activates endothelial cell proliferation and migration, hereby promoting angiogenesis by producing NO (28). Consequently, adequate NO formation prevents endothelial dysfunction, atherosclerosis, and subsequent tissue ischemia. For example, patients with ischemic heart disease may use a NO donor, nitroglycerin, to improve blood flow and endothelial function in ischemic tissue.

NO derived from nNOS has a variety of different functions in the nervous system, including a role as a central regulator of blood pressure and as a neurotransmitter in mediating long-term synaptic transmission. nNOS is expressed in nerves innervating peripheral smooth muscle cells for neurotransmission (29).

Besides arginine, NO derived from arginine is also an important molecule in host defense mechanisms, since it plays a signaling role in the activation of damage-repair mechanisms of the cell. Furthermore, is is a cytotoxic agent and an immune function regulator. NO can initiate apoptosis in hostile cells by activating or inhibiting the expression of genes involved in programmed cell death (30). iNOS is the main NO generating enzyme involved in the immunological effects of NO. Many cells can express iNOS in reaction to inflammatory factors, including immune cells (dendritic cells, monocytes, macrophages, eosinophils, neutrophils, Kupffer cells, natural killer cells), as well as other cell types (fibroblasts, endothelial and epithelial cells, keratinocytes, hepatocytes, chondrocytes, etc.) (31). NO regulates the functional activity, growth and elimination of many immune and inflammatory cells, suggesting also a role in the specific immune response. NO regulates cytokine activation and receptor presentation in these cells. However, the direct effects of NO are non-specific because it does not act through a membrane receptor (29). Actions of NO do not depend primarily on the enzymatic

source, but rather on the cellular context, NO concentration (dependent on the distance from NO source) and initial programming of target cells (31). Significant high amounts of NO can be secreted by immune cells, like macrophages, leading to damage to microbes, parasites, or tumor cells. However, when released at the wrong site, this cytotoxic capacity may also harm healthy cells (29). Furthermore, when high amounts of NO are constantly induced and arginine levels become depleted, NO is relatively instable, which may lead to the formation of radical nitrogen species, like peroxynitrite (32).

Arginine deficiency states

Since arginine and NO derived from arginine are essential in so many ways, an arginine deficiency has the potential to disturb many cellular and organ functions. An arginine deficiency may be the result of 1) a dietary arginine deficiency, 2) increased arginine catabolism, and 3) a decreased rate of endogenous arginine production (22). Dietary arginine deficiency will not be outlined here. Several pathological conditions have been found to induce an arginine deficiency, both through a higher demand and breakdown of arginine and lower substrate levels for arginine *de novo* synthesis (33-36).

As described, arginase is the most important arginine-catabolizing enzyme able to decrease arginine availability. Arginase is expressed in both tissue and plasma, and increased arginase activity is observed in multiple pathological conditions, prominently in patients after surgery or trauma, during inflammation and infection and in patients with cancer (22). Furthermore, arginase activity stimulates intracellular arginine transport into arginase expressing cells (37). This subsequently leads to lower arginine availability. The increased arginase expression hereby also influences the production of NO, because, although the affinity of NOS for arginine is 1000-fold greater than the affinity of arginase, the maximal velocity of arginase is 1000-fold greater than that of the NOS enzymes (3). One can imagine, that the increase in arginine catabolism and subsequently decreased arginine levels and NO production, results in an impairment of immune functions, disbalance in inflammation, vascular and hemodynamic complications, alterations in protein turnover, etc (22,38).

Moreover, in disease states, patients are metabolically stressed, and *de novo* arginine synthesis is failing to meet the needs of the body, and arginine becomes an essential amino acid. Also, other nutrients become scarce and specific (inter) organ metabolism changes during catabolic states (11,39,40). Especially in disease states, the various functions and the potential therapeutic targets of arginine and NO are of interest. In this thesis, the arginine/NO metabolism in surgical oncology is the focal point.

Cancer

Cancer is a major health problem all over the world and the incidence and burden of this malignant disease is progressively increasing. The amount of new patients diagnosed with cancer is still increasing by approximately 1% per year and one in 3 deaths in the Netherlands is due to cancer (41). Cancer is a group of diseases characterized by unregulated cell growth of 'abnormal cells', invasion in surrounding tissue and the tendency to metastasize. The causes of cancer are diverse and complex, and remain only partially understood. The development of cancer is multifactorial and both external factors (tobacco, certain types of infectious agents, radiation, diet, chemicals, etc.) and internal factors (inherited diseases, immune function, hormonal status, genetic mutations, etc) are involved in the onset of any type of cancer. These factors play a role together or in a sequence in the multistep process of cancer development, called the carcinogenesis (Figure 2).

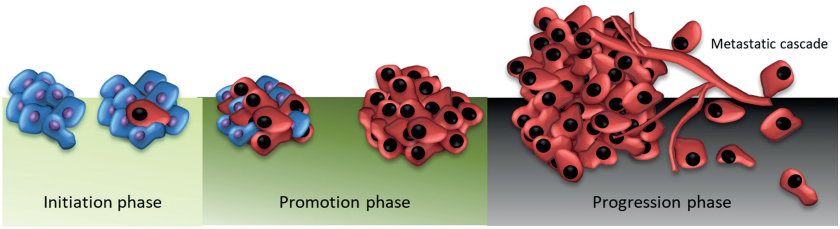

FIGURE 2 | Carcinogenesis.

In the human body, cells are constantly dividing and growing to preserve tissue function. This process is controlled by multiple regulatory mechanisms to ensure development of healthy new cells and elimination of damaged and outdated cells. On a molecular level, cell damage happens almost one million times a day, however in physiological circumstances the regulatory mechanisms are perfectly capable in counteracting these errors. A cancer cell may develop when the cellular genome that affects the expression and function of genes regulating cell growth and differentiation is altered. This first step in the carcinogenesis is the initiation phase of cancer development. Once a cancer cell is instantiated, the immune system is the major surveillant of malignant outgrow (42). The immune system selectively identifies cancer cells by the recognition of expression of tumor-specific antigens and destroys them before they can evolve. Accordingly, multiple perturbations in cellular damage-repair mechanism and in immune surveillance have to occur to develop cancer. Cancer cells need to have six hallmark capabilities to grow out as a malignant tumor: they sustain proliferative signaling, evade growth suppressors, resist cell death, enable replicative immortality, activate invasion and angiogenesis (43). These capabilities lead to the outgrow of a malignant tumor in the promotion phase of the carcinogenesis and to tumor invasion in the surrounding tissue and organs in the progression phase. Additionally, cancer cells can spread throughout the body via the bloodstream and lymphatic system and grow out to distant metastases.

Cancer and the immune function

As described above, the human body has an advanced immune system potentially able to fight cancer. Many types of malignant cells have typical antigens that distinguish them from normal cells. Activated immune cells can specifically identify these targets and eliminate the cells that carry them, hereby preventing malignant outgrow.

It is evident that an inflammatory environment is essential for development of all tumors. For some cancer types, the relationship between inflammation and the initiation of carcinogenesis is very direct (44). For example, chronic infection with hepatitis B or C virus increase the risk of hepatocellular carcinoma (45) and persistent human papilloma virus infection is the underlying cause of cervical cancer (46). This chronic inflammation is primarily the result of protective mechanisms in order to clear these pathogens. Although these mechanisms

are self-limiting in general, a disbalance in inflammatory mediators may lead to pathogenesis and disease progression. Also in cancers without a direct detectable infectious cause, inflammation has been shown to play a central role in tumor development. Physiologically, there is a balance in the expression of pro-inflammatory cytokines (Th1) and anti-inflammatory cytokines (Th2) by T-helper cells (47). When a dysregulation in the expression of inflammatory factors occurs, these factors are able to support angiogenesis, tumor growth and tumor-specific immune suppression (48).

One of the immune suppressing strategies of cancer is the inflow of a class of innate immune cells that help sabotage the anti-tumor immune attack of the host. These immature cells are primarily recruited to control tumor growth, however the disbalance in the production of immune modulating factors in the tumor environment causes the development of a tumor-promoting phenotype. These so called myeloid-derived suppressor cells (MDSC) accumulate in the tumor environment, lymph nodes, spleen and liver of the tumor-bearing host (49,50). The MDSC have many ways of blocking both innate and adaptive immune responses (50-52). They are able to produce the two most important enzymes involved in arginine catabolism: arginase I and iNOS (53,54). Already during carcinogenic initiation, MDSC are found in the host (55). The expression of the enzymes by the MDSC is regulated by the expression of cytokines by T-helper cells. Th1 cytokines promote iNOS expression; Th2 cytokines are anti-inflammatory and induce arginase expression (56). It is suggested that in the first phase of tumor development the MDSC predominantly produce arginase induced by Th2 cytokines. This results in a high level of arginine catabolism, which causes an arginine deficiency (57,58). T-lymphocytes depend on arginine for proliferation and receptor production and therefore depleted arginine levels prevent these immune cells from fighting malignant outgrowth. During further development of the malignant tumor, the Th1/Th2 balance switches to an increase in Th1 cytokine production, promoting the MDSC to express iNOS (56,57). This results in a continuous production of NO in the tumor environment, promoting angiogenesis (see next paragraph) and tumor growth. Furthermore, together high iNOS activity and low arginine levels may lead to the formation of reactive nitrogen species, such as peroxynitrite. These highly reactive molecules damage the surrounding tissue promoting tumor outgrow and also cause immune suppression by blocking the binding of tumor-antigen to T-cell receptors (56).

Cancer and angiogenesis

Malignant tumors cannot grow beyond a certain size or metastasize without blood supply, because non-vascularized tumor tissue will become ischemic and necrotic. The process in which the tumor stimulates migration, growth and differentiation of endothelial cells from the already existing vascular system, forming new blood vessels into the tumor, is called angiogenesis. Angiogenesis follows a pathway that is triggered by the expression of specific growth factors. The best characterized and most important factor is vascular endothelial growth factor (VEGF) (59). One of the most potent stimuli inducing VEGF production is a lack of oxygen in the tumor, commonly referred to as hypoxia (60). Physiologically, cells in a hypoxic environment attempt to re-establish the oxygen and nutrients supply by stimulating the development of new vasculature. The lack of oxygen stimulates the formation of hypoxia induced factor (HIF), which in its turn augments the release of VEGF into the surrounding tissue. The surrounding endothelial cells are stimulated to migrate to the source of VEGF expression to initiate the formation of angiogenetic sprouting (60). Several anti-angiogenetic treatments, targeting the VEGF pathway, have shown promising results in the treatment of various solid malignancies (61).

Nitric oxide is known for its role as an important regulator of vascular homeostasis, including modulation of vascular tone, regulation of endothelial integrity and cell growth. As described, NO is synthesized by the NOS enzymes from arginine. eNOS, that is mainly present in blood vessels, is the primary generator of NO in the endothelium and regulates vascular function and maturation (62). eNOS activity is modulated by oxygen abundance and hypoxia induced VEGF stimulates NO production via eNOS. NO from eNOS is the final mediator of VEGF dependent angiogenesis (63). Also iNOS has a profound role in hypoxia induced angiogenesis. iNOS expression is promoted by (pro)-inflammatory cytokines and molecules induced by a disordered tumor environment, for example tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and HIF (64). The amount NO produced by iNOS is approximately one thousand-fold higher than produced by nNOS or eNOS (64). Many cancer cells express all three isoforms of NOS, with the predominance of iNOS. Similar to the pro-angiogenetic effect of eNOS induced NO, NO produced by iNOS has been shown to mediate angiogenesis via VEGF (65).

After activation of eNOS and iNOS by VEGF, the upregulation of NO levels in the malignant tissue in turn induces VEGF expression and promotes the proliferation of endothelial cells. Thus, there seems to be a positive feed-back system between NO and VEGF in tumor angiogenesis. By activation of the NOS enzymes, VEGF promotes the endogenous production of NO and therefore induces the ability of NO to activate VEGF and facilitate angiogenesis (62). Furthermore, high levels of VEGF and iNOS significantly correlate with the microvessel density in several solid tumors, and are also considered to be independent prognostic indicators of overall survival (65,66).

However, several studies have reported a conflicting role for NO in tumorgenesis and angiogenesis, since high concentrations of NO may also promote the immune system and cell growth arrest in the tumor and endothelial cells (62). These discrepancies may result from differences in tumor stage and type, host and tumor models in these studies. Thus, the role of NO in tumor biology is complex, because it has both facilitatory and inhibitory roles in malignant processes depending on tumor stage, cell type, the local concentration of NO, and the presence of other regulators (67).

As explained, ADMA is an inhibitor of NO formation by all NOS enzymes and hereby regulates NO bioavailability. Only a few studies analyzed the role of ADMA and DDAH in tumor angiogenesis, however the role of ADMA and DDAH in angiogenesis in cardiovascular disease is extensively studied (68). In cardiovascular disease, high ADMA levels are a risk factor for deficient vascular function because it impairs NO mediated angiogenesis. Contrarily, high DDAH levels, the degrading ADMA enzyme, may maintain NO bioavailability and hereby promote angiogenesis (69). Indeed, DDAH overexpression is associated with increased VEGF expression. One of the few studies on tumor angiogenesis showed that NOS, DDAH and VEGF expression is increased in a prostate cancer cell line compared to benign hypertrophic prostate cells (70). Another study showed that overexpression of DDAH in malignant brain tumors is stimulated by hypoxia and results in blood vessel formation, increased blood content in the tumor and even tumor growth (71,72).

Cancer cachexia

Involuntary weight loss is seen in the majority of patients with advanced malignant disease, which can be due to anorexia, reduced appetite, treatment-related malnutrition and also the catabolic state of cachexia. The word cachexia is derived from the Greek words *kakos* (bad) and *hexis* (condition). More than two thousand years ago, Hippocrates described a syndrome of wasting and progressive weakness in ill and dying patients. Cachexia, is a complex metabolic syndrome associated with underlying illness and characterized by loss of muscle mass. Cachexia causes weight loss and anorexia, inflammation, insulin resistance, and increased muscle protein breakdown are frequently associated with cachexia. Cachexia is distinct from starvation and is associated with increased morbidity (73,74).

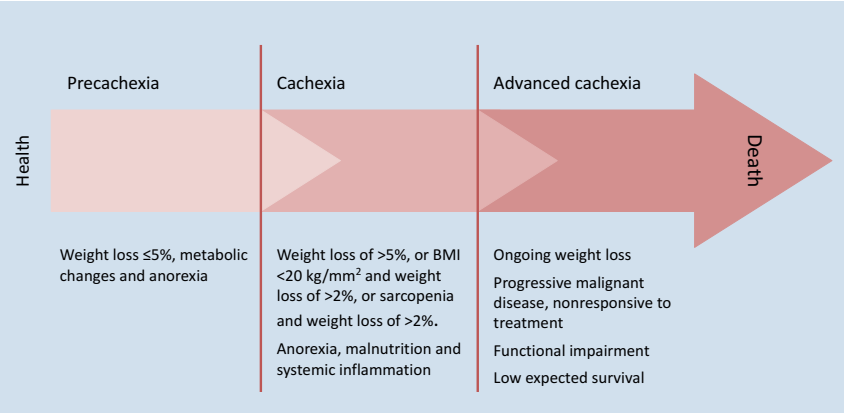

FIGURE 3 | Stages of cancer cachexia. Not all patients will be subjected to this whole spectrum of cachexia.

Cancer cachexia is an ongoing process and can be categorized in three stages of clinical relevance (Figure 3) (74). The rate of progression in this spectrum depends on patient characteristics, but also on type of malignancy and therapy compliance. Precachexia is characterized by involuntary weight loss caused by early clinical and metabolic signs, such as anorexia and decreased glucose tolerance. Cachexia is the stage where patients show more than 5% loss of body weight over the past 6 months, or a body mass index of less than 20 $kg \cdot m^{-2}$ and proceeding weightloss of >2%, or sarcopenia and proceeding weightloss of

>2%. In this stage, patient and tumor-derived mechanisms cause ongoing loss of body weight and skeletal muscle that cannot be (fully) reversed by conventional nutritional support and leads to functional impairment. In advanced or refractory cachexia, the hypercatabolic state as a result of advanced cancer or rapidly progressive cancer is clinically persistent. Although lots of information on the pathophysiology of cachexia is gained in the last decade, the main therapeutic interventions are based on symptom control and prevent the complications of cachexia instead of counteracting the underlying mechanisms.

Approximately half of all cancer patients show signs of cachexia as described above, however the incidence of the development of cachexia depends on the type of malignancy. Cachexia is characterized by depletion of lean body mass and therefore distinguished from starvation, which induces a metabolic shift in energy source from glucose to fat and ketones, leading to preservation of the muscle protein compartment (75). Cancer cachexia is a multifactorial process in which systemic inflammation, anorexia and a disturbed metabolism contribute to sarcopenia and reduction in body weight. Importantly, cachexia and malnutrition result in impaired immunity itself. In cachexia, the balance in protein synthesis and protein breakdown is disturbed, and the arginine/NO pathway has been shown to be of great importance in preserving this balance (76-78). Multiple cytokines have been postulated to play a role in the etiology of this hypercatabolic state, in which protein synthesis is diminished and protein breakdown is accelerated. Pro-inflammatory cytokines TNF-α, IL-1β, IL-6, IL-8 and IFN-γ are so called pro-cachectic mediators, which are activated by tumor cells and host (immune) cells and lead to a progressive cachectic state (79). For example TNF-α augments gluconeogenesis, lipolysis and proteolysis and diminishes protein synthesis. It also stimulates the expression of other pro-cachectic cytokines as IL-1β (80). TNF-α and IL-1β both increase energy expenditure, protein breakdown and loss of body weight, furthermore they have been suggested to induce cancer related anorexia by direct and indirect inhibiting influences on food intake (79). Furthermore, IL-6 levels are elevated in patients with a muscle wasting syndrome and increasing IL-6 levels correlate with the development of cancer cachexia. Skeletal muscle exposure to IL-6 induces protein degradation pathways, leading to wasting (81). The arginine/NO pathway plays a regulating role in the inflammatory catabolic state induced by these cytokines. Decreased arginine levels are associated with the stimulation of the expression of several pro-cachectic cytokines, such as TNF-α, IL-6 and IL-8 (82,83). However, it was shown that arginine supplementation is able to decrease levels of TNF-α and IL-6 (84).

Cancer and surgery

As described above cancer goes hand in hand with a tumor-induced disbalance in the host immune function. Cancer is a heterogeneous group of diseases with multiple causes, however the involvement of the immune system is a common denominator (85,86). Both the inflammatory state and the catabolic state of cancer patients result in a vulnerable physical condition and makes cancer patients less resistant to physical stress. Undergoing surgery induces traumatic stress responses, and a low fitness due to cancer is not a good starting point. This may lead to an increased complication risk (87). However, in most types of malignancies, surgery is still implemented in the current oncological treatment strategies.

Surgery induces a variety of stress responses and hereby leads to a disturbance in the immune function (88). The postoperative period is characterized by a generalized state of immune depression, just at the time that risk on invasion of pathogens and tumor cells is increased. This makes the patient particular vulnerable for infectious complications and tumor metastasis and proliferation during this postoperative period. Local production of inflammatory mediators at the site of the operation may lead to a systemic inflammatory immune response (SIRS), which initially is considered to be physiological and necessary for the elimination of pathogens, reduce tissue damage and improve recovery. Moreover, the body has a compensatory mechanism to maintain homeostasis in the postoperative period by activating the production of anti-inflammatory mediators (89). However, persistent disbalance in the immune system, due to pre-existent conditions such as cancer and malnutrition or postoperative complications, may lead to immune suppression. As described, bearing cancer results in a disturbance in the Th1/Th2 balance of the immune system. After surgery, the immune response shifts to a domination of Th2 response and a decrease of the Th1 response, leading to an impaired cell mediated immunity, which is important for tumor cell elimination (90). Furthermore, this shift also results in the promotion of catabolism of arginine by arginase. This is supported by studies showing a decrease in arginine levels in patients just a few hours after surgical injury, indicating an increased breakdown of arginine rather than a deficient intake (91). The arginine deficiency state after surgery could potentially be treated with arginine supplementation. Most studies investigating the effect of arginine enriched nutrition perioperatively are conducted in patients bearing cancer and in international guidelines on the role of immune nutrition in surgical patients arginine administration (in combination with other immune enhancing

agents) is particularly recommended in cancer patients undergoing major elective surgery (92). One can imagine that the double immune dysfunction, caused by surgery and the malignant disease, gains the most effect from (nutritional) interventions counteracting a perturbation of the arginine metabolism.

Arginine supplementation in surgical oncology

As outlined, arginine supplementation in cancer patients undergoing surgery has the potential to improve immune function and thereby decreases postoperative complications and improves outcome. Reduced plasma arginine levels are found in various types of cancer, including breast, colon, pancreas, head- and neck, and lung cancer (36,93,94). This arginine deficiency is irrespective of weight loss, tumor stage or body mass index, suggesting a general reduced arginine availability in the cancer bearing state. Immune-enhancing nutritional interventions enriched with arginine may restore arginine levels and thereby immune function. Arginine supplementation corrects the Th1/Th2 imbalance in patients undergoing oncological surgery and enhances the innate and adaptive immune responses (90). Furthermore, arginine administration has been shown to improve oncological outcomes in multiple experimental settings (95-97). Several meta-analyses showed a significant better outcome, in terms of postoperative complications, postoperative hospital stay and even improved survival (98-100). Most of the included studies in these meta-analyses used enteral formulas containing multiple immune modulating nutrients (*n*-3 fatty acids, arginine, glutamine, nucleotides), leaving it indecisive which of the individual nutrients or mixture was responsible for the improvement in outcomes. International guidelines recommend the administration of arginine as part of an immune modulating nutritional intervention in cancer patients undergoing major elective surgery (92,101). One can also hypothesize that glutamine supplementation, the precursor of arginine via the intestinal-renal axis, may improve endogenous arginine synthesis in cancer patients in the perioperative phase. It was shown that glutamine supplementation indeed may reduce complications after chemotherapy and surgery in patients with colorectal and lung cancer (102,103).

Although these results sound promising for improving perioperative care in cancer patients and thereby reducing postoperative complications, the role of arginine supplementation alone in cancer patients remains controversial. Various tumor cells have been shown to be auxotrophic for arginine, which indicates that these malignant cells have a deficiency in arginine synthesizing enzymes as ASS and

can only survive and expand invasively if arginine is available (104). In these tumor types, arginine supplementation is suggested to be contra-indicated and arginine deprivation by arginine catabolizing enzymes could be a potential strategy for the treatment of these malignancies (105,106). Hence, this makes the application of arginine and NO targeting strategies in surgical oncology complex and ambiguous. In this thesis, we try to clarify this complex metabolism.

Aim and outline of the thesis

The general aim of this thesis is to elucidate the role of the arginine/NO pathway in cancer development and to study the effect of arginine enhancing diets on metabolic and clinical outcomes in surgical cancer patients.

In **PART I** of this thesis, the interorgan metabolism of arginine and NO is investigated and their role in clinical nutrition is identified. **CHAPTER TWO** describes a review to outline the potential of the administration of nutritional substrates, including arginine and its precursors, to surgical patients and the underlying mechanisms that make them particularly important in perioperative care. **CHAPTER THREE** and **CHAPTER FOUR** outline the newly developed analysis method and the results of a metabolic study using a stable isotope technique on the qualitative and quantitative effects of an intravenous supplement of glutamine on renal *de novo* arginine synthesis in humans. In **CHAPTER FIVE** the main findings of the studies of **PART I** are clarified and future scientific perspectives are described.

PART II of this thesis focuses on arginine/NO metabolism in surgical oncology in particular. **CHAPTER SIX** is a detailed overview of our hypothesis on the role of the arginine/NO pathway in the onset of cancer cachexia. **CHAPTER SEVEN** describes a metabolic study in tumor-bearing rats, determining the effect of cancer on the interorgan pathway of glutamine, citrulline and arginine. In this study, we also present the effects of a glutamine enriched diet on this pathway in rats with cancer. In **CHAPTER EIGHT** we describe the long-term clinical outcome of a double blind randomized clinical trial on a perioperative arginine enriched diet in malnourished head and neck cancer patients. Alterations in the arginine/NO pathway on cellular and tissue level in human primary liver cancer and the effect of these alterations on tumor angiogenesis are studied in **CHAPTER NINE**. In **CHAPTER TEN** the main findings of the studies described in **PART II** are discussed and future scientific perspectives are proposed.

References

1. Wu G, Bazer FW, Davis TA et al. Arginine metabolism and nutrition in growth, health and disease. Amino Acids 2009;37(1):153-168.
2. Morris SM, Jr. Arginine metabolism: boundaries of our knowledge. J Nutr 2007;137(6 Suppl 2):1602S-1609S.
3. Wu G, Morris SM, J. Arginine metabolism: nitric oxide and beyond. Biochem J 1998;336(Pt 1): 1–17.
4. Morris SM, Jr. Regulation of enzymes of the urea cycle and arginine metabolism. Annu Rev Nutr 2002;22:87-105.
5. Suchner U, Heyland DK, Peter K. Immune-modulatory actions of arginine in the critically ill. Br J Nutr 2002;87 Suppl 1:S121-S132.
6. Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. Nat Rev Immunol 2005;5(8):641-654.
7. Popovic PJ, Zeh HJ, Ocho JB. Arginine and immunity. J Nutr. 2007;137(6 Suppl 2):1681S-1686S.
8. Castillo L, Beaumier L, Ajami AM, Young VR. Whole body nitric oxide synthesis in healthy men determined from [15N] arginine-to-[15N]citrulline labeling. Proc Natl Acad Sci U S A 1996;93(21):11460-11465.
9. Visek WJ. Arginine needs, physiological state and usual diets. A reevaluation. J Nutr 1986;116(1):36-46.
10. Morris SM, Jr. Arginine: beyond protein. Am J Clin Nutr 2006;83(2):508S-512S.
11. Ligthart-Melis GC, van de Poll MC, Boelens PG, Dejong CH, Deutz NE, van Leeuwen PA. Glutamine is an important precursor for *de novo* synthesis of arginine in humans. Am J Clin Nutr 2008;87(5):1282-1289.
12. Windmueller HG, Spaeth AE. Intestinal metabolism of glutamine and glutamate from the lumen as compared to glutamine from blood. Arch Biochem Biophys 1975;171(2):662-672.
13. Deutz NE. The 2007 ESPEN Sir David Cuthbertson Lecture: amino acids between and within organs. The glutamate-glutamine-citrulline-arginine pathway. Clin Nutr 2008;27(3):321-327.
14. Crenn P, Coudray-Lucas C, Thuillier F, Cynober L, Messing B. Postabsorptive plasma citrulline concentration is a marker of absorptive enterocyte mass and intestinal failure in humans. Gastroenterology 2000;119(6):1496-1505.
15. Rouge C, Des RC, Robins A et al. Manipulation of citrulline availability in humans. Am J Physiol Gastrointest Liver Physiol 2007;293(5):G1061-G1067.
16. van de Poll MC, Ligthart-Melis GC, Boelens PG, Deutz NE, van Leeuwen PA, Dejong CH. Intestinal and hepatic metabolism of glutamine and citrulline in humans. J Physiol 2007;581(Pt 2):819-827.
17. Morris SM, Jr., Sweeney WE, Jr., Kepka DM, O'Brien WE, Avner ED. Localization of arginine biosynthetic enzymes in renal proximal tubules and abundance of mRNA during development. Pediatr Res 1991;29(2):151-154.
18. Closs EI, Simon A, Vécony N, Rotmann A. Plasma membrane transporters for arginine. J Nutr 2004;134(10 Suppl):2752S-2759S.
19. White MF. The transport of cationic amino acids across the plasma membrane of mammalian cells. Biochim Biophys Acta 1985;822(3-4):355-374.
20. Deignan JL, Livesay JC, Yoo PK et al. Ornithine deficien cy in the arginase double knockout mouse. Mol Genet Metab 2006;89(1-2):87-96.
21. Morris SM, Jr. Enzymes of arginine metabolism. J Nutr 2004 Oct;134(10 Suppl):2743S-2747S.
22. Morris SM, Jr. Arginases and arginine deficiency syndromes. Curr Opin Clin Nutr Metab Care 2012;15(1):64-70.

23. Moncada S. The L-arginine-nitric oxide pathway. N Engl J Med 1993;329(27):2002-12.
24. Burke AJ, Sullivan FJ, Giles FJ, Glynn SA. The Yin and Yang of Nitric Oxide in Cancer Progression. Carcinogenesis 2013;34(3):503-512.
25. Bansal V, Ochoa JB. Arginine availability, arginase, and the immune response. Curr Opin Clin Nutr Metab Care 2003;6(2):223-228.
26. Boger RH. Asymmetric dimethylarginine, an endogenous inhibitor of nitric oxide synthase, explains the "L-arginine paradox" and acts as a novel cardiovascular risk factor. J Nutr 2004;134(10 Suppl):2842S-2847S.
27. Fiedler LR, Wojciak-Stothard B.. The DDAH/ADMA pathway in the control of endothelial cell migration and angiogenesis. Biochem Soc Trans 2009;37(Pt 6):1243-1247.
28. Tousoulis D, Kampoli AM, Tentolouris C, Papageorgiou N, Stefanadis C. The role of nitric oxide on endothelial function. Curr Vasc Pharmacol 2012;10(1):4-18.
29. Forstermann U, Sessa WC. Nitric oxide synthases: regulation and function. Eur Heart J 2012;33(7):829-837d.
30. Nicotera P, Bonfoco E, Brüne B. Mechanisms for nitric oxide-induced cell death: involvement of apoptosis. Adv Neuroimmunol 1995;5(4):411-420.
31. Bogdan C. Nitric oxide synthase in innate and adaptive immunity: an update. Trends Immunol 2015;36(3):161-178.
32. Matata BM. Peroxynitrite is an essential component of cytokines production mechanism in human monocytes through modulation of nuclear factor-kappa B DNA binding activity. 2002.
33. Davis JS, Anstey NM. Is plasma arginine concentration decreased in patients with sepsis? A systematic review and meta-analysis. Crit Care Med 2011;39(2):380-385.
34. Luiking YC, ten Have GA, Wolfe RR, Deutz NE. Arginine *de novo* and nitric oxide production in disease states. Am J Physiol Endocrinol Metab 2012;303(10):E1177-E1189.
35. Schramm L, La M, Heidbreder E et al. L-arginine deficiency and supplementation in experimental acute renal failure and in human kidney transplantation. Kidney Int 2002;61(4):1423-1432.
36. Vissers YL, Dejong CH, Luiking YC, Fearon KC, von Meyenfeldt MF, Deutz NE. Plasma arginine concentrations are reduced in cancer patients: evidence for arginine deficiency? Am J Clin Nutr 2005;81(5):1142-1146.
37. Luiking YC, Poeze M, Dejong CH, Ramsay G, Deutz NE. Sepsis: an arginine deficiency state? Crit Care Med 2004;32(10):2135-2145.
38. Tong BC, Barbul A. Cellular and physiological effects of arginine. Mini Rev Med Chem 2004;4(8):823-832.
39. Luiking YC, Poeze M, Ramsay G, Deutz NE. Reduced citrulline production in sepsis is related to diminished *de novo* arginine and nitric oxide production. Am J Clin Nutr 2009;89(1):142-152.
40. Nijveldt RJ, Prins HA, Siroen MP, Rauwerda JA, Teerlink T, van Leeuwen PA. Low arginine plasma levels in patients after thoracoabdominal aortic surgery. Eur J Clin Nutr 2000;54(8):615-617.
41. Integraal Kankercentrum Nederland. Nederlandse Kanker Registratie. 4-2-2016.
42. Swann JB, Smyth MJ. Immune surveillance of tumors. J Clin Invest 2007;117(5):1137-1146.
43. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100(1):57-70.
44. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell 2010;140(6):883-899.

45. Takeda H, Takai A, Inuzuka T, Marusawa H. Genetic basis of hepatitis virus-associated hepatocellular carcinoma: linkage between infection, inflammation, and tumorigenesis. J Gastroenterol 2017;52(1):26-38.
46. Schiffman M, Castle PE, Jeronimo J, Rodriguez AC, Wacholder S. Human papillomavirus and cervical cancer. Lancet 2007;370(9590):890-907.
47. Ostrand-Rosenberg S. Immune surveillance: a balance between protumor and antitumor immunity. Curr Opin Genet Dev 2008;18(1):11-18.
48. de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. Nat Rev Cancer 2006;6(1):24-37.
49. Candido J, Hagemann T. Cancer-related inflammation. J Clin Immunol 2013;33 Suppl 1:S79-S84.
50. Schmid MC, Varner JA. Myeloid cells in the tumor microenvironment: modulation of tumor angiogenesis and tumor inflammation. J Oncol 2010;201026.
51. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol 2009;9(3):162-174.
52. Marx J. Cancer immunology. Cancer's bulwark against immune attack: MDS cells. Science 2008;319(5860):154-156.
53. Mazzoni A, Bronte V, Visintin A et al. Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. J Immunol 2002;168(2):689-695.
54. Rodriguez PC, Ochoa AC. Arginine regulation by myeloid derived suppressor cells and tolerance in cancer: mechanisms and therapeutic perspectives. Immunol Rev 2008;222:180-191.
55. Winfield RD, Delano MJ, Pande K, Scumpia PO, Laface D, Moldawer LL. Myeloid-derived suppressor cells in cancer cachexia syndrome: a new explanation for an old problem. JPEN J Parenter Enteral Nutr 2008;32(6):651-655.
56. Bronte V, Serafini P, Mazzoni A, Segal DM, Zanovello P. L-arginine metabolism in myeloid cells controls T-lymphocyte functions. Trends Immunol 2003;24(6):302-306.
57. Redente EF, Orlicky DJ, Bouchard RJ, Malkinson AM. Tumor signaling to the bone marrow changes the phenotype of monocytes and pulmonary macrophages during urethane-induced primary lung tumorigenesis in A/J mice. Am J Pathol 2007;170(2):693-708.
58. Rodriguez PC, Quiceno DG, Zabaleta J et al. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. Cancer Res 2004;64(16):5839-49.
59. Kerbel RS. Tumor angiogenesis. N Engl J Med 2008;358(19):2039-2049.
60. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003;3(10):721-732.
61. Jayson GC, Kerbel R, Ellis LM, Harris AL. Antiangiogenic therapy in oncology: current status and future directions. Lancet 2016;388(10043):518-529.
62. Fukumura D, Kashiwagi S, Jain RK. The role of nitric oxide in tumour progression. Nat Rev Cancer 2006;6(7):521-534.
63. Duda DG, Fukumura D, Jain RK. Role of eNOS in neovascularization: NO for endothelial progenitor cells. Trends Mol Med 2004;10(4):143-145.
64. Singh S, Gupta AK. Nitric oxide: role in tumour biology and iNOS/NO-based anticancer therapies. Cancer Chemother Pharmacol 2011;67(6):1211-1224.
65. Song ZJ, Gong P, Wu YE. Relationship between the expression of iNOS, VEGF, tumor angiogenesis and gastric cancer. World J Gastroenterol 2002;8(4):591-595.
66. Wesseling P, Ruiter DJ, Burger PC. Angiogenesis in brain tumors; pathobiological and clinical aspects. J Neurooncol 1997;32(3):253-265.

67. Vannini F, Kashfi K, Nath N. The dual role of iNOS in cancer. Redox Biol 2015;6:334-343.
68. Fiedler LR, Bachetti T, Leiper J et al. The ADMA/DDAH pathway regulates VEGF-mediated angiogenesis. Arterioscler Thromb Vasc Biol. 2009;29(12):2117-2124.
69. Zhang P, Xu X, Hu X et al. DDAH1 Deficiency Attenuates Endothelial Cell Cycle Progression and Angiogenesis. PLoS One 2013;8(11):e79444.
70. Vanella L, Di Giacomo C, Acquaviva R et al. The DDAH/NOS pathway in human prostatic cancer cell lines: Antiangiogenic effect of L-NAME. Int J Oncol 2011;39(5):1303-1310.
71. Kostourou V, Robinson SP, Cartwright JE, Whitley GS. Dimethylarginine dimethylaminohydrolase I enhances tumour growth and angiogenesis. Br J Cancer 2002;87(6):673-680.
72. Kostourou V, Robinson SP, Whitley GS, Griffiths JR. Effects of overexpression of dimethylarginine dimethylaminohydrolase on tumor angiogenesis assessed by susceptibility magnetic resonance imaging. Cancer Res 2003;63(16):4960-4966.
73. Evans WJ, Morley JE, Argiles J et al. Cachexia: a new definition. Clin Nutr 2008;27(6):793-799.
74. Fearon K, Strasser F, Anker SD et al. Definition and classification of cancer cachexia: an international consensus. Lancet Oncol 2011;12(5):489-495.
75. Tisdale MJ. Cancer cachexia. Curr Opin Gastroenterol 2010;26(2):146-151.
76. Hall DT, Ma JF, Marco SD, Gallouzi IE. Inducible nitric oxide synthase (iNOS) in muscle wasting syndrome, sarcopenia, and cachexia. Aging 2011;3(8):702-715.
77. Jobgen WS, Fried SK, Fu WJ, Meininger CJ, Wu G. Regulatory role for the arginine-nitric oxide pathway in metabolism of energy substrates. J Nutr Biochem 2006;17(9):571-588.
78. Yao K, Yin YL, Chu W et al. Dietary arginine supplementation increases mTOR signaling activity in skeletal muscle of neonatal pigs. J Nutr 2008;138(5):867-72.
79. Argiles JM, Busquets S, Toledo M, Lopez-Soriano FJ. The role of cytokines in cancer cachexia. Curr Opin Support Palliat Care 2009;3(4):263-268.
80. Tijerina AJ. The biochemical basis of metabolism in cancer cachexia. Dimens Crit Care Nurs 2004;23(6):237-243.
81. Narsale AA, Carson JA. Role of interleukin-6 in cachexia: therapeutic implications. Curr Opin Support Palliat Care 2014;8(4):321-327.
82. Breuillard C, Belabed L, Bonhomme S et al. Arginine availability modulates arginine metabolism and TNFalpha production in peritoneal macrophages from Zucker Diabetic Fatty rats. Clin Nutr 2012;31(3):415-421.
83. Fan XY, van den Berg A, Snoek M et al. Arginine deficiency augments inflammatory mediator production by airway epithelial cells *in vitro*. Respir Res 2009;10:62.
84. Chen DW, Wei FZ, Zhang YC, Ou JM, Xu J. Role of enteral immunonutrition in patients with gastric carcinoma undergoing major surgery. Asian J Surg 2005;28(2):121-124.
85. Herber DL, Nagaraj S, Djeu JY, Gabrilovich DI. Mechanism and therapeutic reversal of immune suppression in cancer. Cancer Res 2007;67(11):5067-5069.
86. Marigo I, Dolcetti L, Serafini P, Zanovello P, Bronte V. Tumor-induced tolerance and immune suppression by myeloid derived suppressor cells. Immunol Rev 2008 Apr;222:162-179.
87. Evans C, Dalgleish AG, Kumar D. Review article: immune suppression and colorectal cancer. Aliment Pharmacol Ther 2006;24(8):1163-1177.
88. Lennard TW, Shenton BK, Borzotta A et al. The influence of surgical operations on components of the human immune system. Br J Surg 1985;72(10):771-776.

89. Marik PE, Flemmer M. The immune response to surgery and trauma: Implications for treatment. J Trauma Acute Care Surg 2012;73(4):801-808.

90. Matsuda A, Furukawa K, Takasaki H et al. Preoperative oral immune-enhancing nutritional supplementation corrects TH1/TH2 imbalance in patients undergoing elective surgery for colorectal cancer. Dis Colon Rectum 2006;49(4):507-516.

91. Ochoa JB, Bernard AC, O'Brien WE et al. Arginase I expression and activity in human mononuclear cells after injury. Ann Surg 2001;233(3):393-9.

92. Weimann A, Braga M, Carli F et al. ESPEN Guidelines Clinical Nutrition in surgery. Clin Nutr 2017;36:623-650.

93. Naini AB, Dickerson JW, Brown MM. Preoperative and postoperative levels of plasma protein and amino acid in esophageal and lung cancer patients. Cancer 1988;62(2):355-360.

94. Schlemmer M, Suchner U, Schapers B et al. Is glutamine deficiency the link between inflammation, malnutrition, and fatigue in cancer patients? Clin Nutr 2015;34(6):1258-1265.

95. Cao Y, Feng Y, Zhang Y, Zhu X, Jin F. L-Arginine supplementation inhibits the growth of breast cancer by enhancing innate and adaptive immune responses mediated by suppression of MDSCs *in vivo*. BMC Cancer 2016;16:343.

96. Ma Q, Williamson KE, O'rourke D, Rowlands BJ. The effects of l-arginine on crypt cell hyperproliferation in colorectal cancer. J Surg Res 1999;81(2):181-188.

97. Zhao H, Zhao H, Wang Y, Jing H, Ding Q, Xue J. Randomized clinical trial of arginine-supplemented enteral nutrition versus standard enteral nutrition in patients undergoing gastric cancer surgery. J Cancer Res Clin Oncol 2013;139(9):1465-1470.

98. Marimuthu K, Varadhan KK, Ljungqvist O, Lobo DN. A meta-analysis of the effect of combinations of immune modulating nutrients on outcome in patients undergoing major open gastrointestinal surgery. Ann Surg 2012;255(6):1060-1068.

99. Vidal-Casariego A, Calleja-Fernandez A, Villar-Taibo R, Kyriakos G, Ballesteros-Pomar MD. Efficacy of arginine-enriched enteral formulas in the reduction of surgical complications in head and neck cancer: a systematic review and meta-analysis. Clin Nutr 2014;33(6):951-957.

100. Wong CS, Aly EH. The effects of enteral immunonutrition in upper gastrointestinal surgery: A systematic review and meta-analysis. Int J Surg 2016;29:137-150.

101. Arends J, Bachmann P, Baracos V et al. ESPEN guidelines on nutrition in cancer patients. Clin Nutr. 2017;36(1):11-48.

102. Jolfaie NR, Mirzaie S, Ghiasvand R, Askari G, Miraghajani M. The effect of glutamine intake on complications of colorectal and colon cancer treatment: A systematic review. J Res Med Sci 2015;20(9):910-918.

103. Gul K, Mehmet K, Meryem A. The effects of oral glutamine on clinical and survival outcomes of non-small cell lung cancer patients treated with chemoradiotherapy. Clin Nutr 2016.

104. Qiu F, Huang J, Sui M. Targeting arginine metabolism pathway to treat arginine-dependent cancers. Cancer Lett 2015;364(1):1-7.

105. Park KG, Heys SD, Blessing K et al. Stimulation of human breast cancers by dietary L-arginine. Clin Sci (Lond) 1992;82(4):413-417.

106. Patil MD, Bhaumik J, Babykutty S, Banerjee UC, Fukumura D. Arginine dependence of tumor cells: targeting a chink in cancer's armor. Oncogene 2016;35(38):4957-4972.

PART I

CLINICAL NUTRITION AND ARGININE/NO METABOLISM

Novel nutritional substrates in surgery

Buijs N
Wörner EA
Brinkmann SJH
Luttikhold J
Van der Meij BS
Houdijk APJ
Van Leeuwen PAM

Published in:
Proceedings of the Nutrition Society 2013; 72: 277-287

abstract

Pharmaco-nutrients have beneficial effects on protective and immunological mechanisms in patients undergoing surgery, which are important for recovery after injury and in combating infectious agents. The aim of this review article was to outline the potential of the administration of nutritional substrates to surgical patients and the underlying mechanisms that make them particularly important in perioperative care. Surgery causes a stress response, which has catabolic effects on the body's substrate stores. The amino acid glutamine is a stimulating agent for immune cells. It activates protective mechanisms through its role as a precursor for antioxidants and it improves the barrier function of the gut. Arginine also enhances the function of the immune system, since it is the substrate for T-lymphocytes. Furthermore, *n*-3 polyunsaturated fatty acids (PUFA) stabilize surgery-induced hyperinflammation. Taurine is another substrate that may counteract the negative effects of surgical injury on acid–base balance and osmotic balance. These pharmaco-nutrients rapidly become deficient under the influence of surgical stress. Supplementation of these nutrients in surgical patients may restore their protective and immune-enhancing actions and improve clinical outcome. Moreover, preoperative fasting is still common practice in the Western world, although fasting has a negative effect on the patient's condition and the recovery after surgery. This may be counteracted by a simple intervention such as administering a carbohydrate-rich supplement just before surgery. In conclusion, there are various nutritional substrates that may be of great value in improving the condition of the surgical patient, which may be beneficial for postoperative recovery.

Introduction

In the last few decades, adequate perioperative care has shown to be of great value in improving clinical outcome in surgical patients. Although the importance of nutritional support is increasingly acknowledged, it is still not incorporated in common perioperative practice. Also, the potential positive effects of specific pharmaco-nutrients for surgical patients have not yet been optimally exploited. A stress response after surgery and the concomitant impaired immune function are important factors that negatively influence clinical outcome. The administration of specific nutritional substrates, such as glutamine, arginine, *n*-3 PUFA and taurine, to surgical patients may balance this surgical stress response and the associated inflammatory reaction, support the cell-mediated immune function and may consequently improve outcome. Despite large amounts of research data on these substrates, the implementation in current clinical practice is disappointing. Also, fasting before surgery is still common practice in preoperative care in many Western countries, even though international guidelines of various professional nutrition societies state that preoperative fasting is unwanted. A simple intervention such as the supplementation of carbohydrates (CHO) just 2h before surgery may improve the metabolic condition of the patient and thereby clinical outcome. The purpose of this article is to review the underlying mechanisms explaining the pharmacological actions of several novel substrates and their potential role in nutritional care in surgical patients.

Glutamine

The immune system is of fundamental importance for the recovery from surgery. It is not only essential in preventing or limiting infections, but also in the overall process of repair and recovery from injury. Glutamine is a conditionally essential amino acid during metabolic stress, induced by major surgery. Glutamine is an important amino acid for the immune system, for the glutathione system and also for gut mucosa integrity.

Background

In immune cells glutamine regulates the inflammatory response and is important for cell proliferation and differentiation (1). Glutamine functions as the primary fuel

for these cells, because it is the substrate for glutamate synthase (NADPH), which is essential for intracellular energy supply. T- and B-lymphocytes are the major components of the adaptive immune system, which prevents and eliminates pathogenic invasion. Extracellular glutamine regulates the proliferation of T-lymphocytes and antigen presentation. B-lymphocyte differentiation is also glutamine dependent and their proliferation rate significantly increases when glutamine levels are increased. Macrophages are immune cells that destroy cellular debris and pathogens; accordingly, to do so, macrophages need glutamine as their energy substrate (2). Furthermore, glutamine depletion limits the activation of lymphokine-activated killer cells, which have a very broad target cell spectrum (3).

Glutamine is important for cell protection against oxidative stress. Firstly, glutamine has a protective capacity due to its role as a substrate for the synthesis of glutathione, the major intracellular antioxidant (4). Glutathione has the ability to counteract oxidative injury caused by oxygen-derived free radicals and peroxides, as seen in surgery. When muscle glutamine concentrations decrease during stress, glutathione depletion may occur (4). However, supplementation of glutamine during surgical stress can sustain adequate glutathione levels (5).

Another mechanism of glutamine against the damaging effects of oxidative stress is its stimulating role in the expression of the tissue heat shock protein 70 (6,7). Heat shock protein 70 is essential for cellular recovery after injury and is protective against tissue damage. Absence of heat shock protein 70 may lead to increased cellular apoptosis.

The gut has an important barrier function with concomitant protection mechanisms, since it is intensively exposed to exogenous pathogens. Following physical stress associated with surgery, the barrier function of the gastro-intestinal tract may be impaired. This loss of barrier function may play a role in the translocation of bacteria and endotoxins across the gut wall, subsequently resulting in a prolonged systemic inflammatory response and sepsis. Glutamine is an important regulator of the intestinal integrity, because it alters the expression of tight junction proteins and improves the epithelial barrier function (8). Glutamine is also utilized as a major fuel and nucleotide substrate by intestinal mucosal cells and the gut-associated lymphoid tissue system (2).

Part of the benefits of glutamine supplementation is a consequence of its role as a precursor for endogenous synthesis of arginine through an intestinal–renal pathway involving interorgan transport of citrulline (9,10). It contributes to a

greater intestinal release of citrulline when given enterally and higher plasma levels of citrulline (11). Also glutamine can serve as a precursor for the production of taurine (12). Arginine and taurine and their role in nutritional care in surgery will be discussed later.

Glutamine supplementation in surgical patients

It is proposed that supplementation of glutamine in surgical patients is important, because it may protect cells against injury and patients against complications associated with the key roles described earlier. Thus, glutamine should be administered to build up sufficient levels in order to sustain an appropriate response to stress or injury and protect the patient against a poor clinical outcome.

Glutamine can be given via either the enteral or parenteral route. In both ways, it is given as a dipeptide; because glutamine itself has limited stability in aqueous solutions, adding alanine or glycine to form a dipeptide makes it easily hydrolyzed and stable.

Delivery of parenteral glutamine raises systemic levels of glutamine more than a similar dose of glutamine given by the enteral route. Although glutamine can maintain gut integrity even when delivered from the vascular side of the intestinal epithelial cell, enteral supplementation is more beneficial in preserving the gut barrier function (13). Furthermore, enteral glutamine supplementation is suggested to contribute more to the *de novo* synthesis of arginine than does parenterally administered glutamine (14).

Parenteral route: Preoperative supplementation. Few studies are available on the effect of glutamine supplementation before surgery. In one study, where glutamine was given 5 days before surgery and was stopped on the day before surgery, no beneficial effects were seen. Despite the fact that the potential effects of glutamine were not sustained after surgery, the preoperative immune indices (leucocytes, granulocytes and lymphocytes) were increased by glutamine supplementation (15).

Parenteral route: Perioperative supplementation. Perioperative glutamine administration is associated with reduced immune suppression, an improved capacity to inactivate endotoxins and a significant increase in CD4+ count (marker of immune cells) after surgery (16,17). In colorectal surgery, perioperative supplementation of glutamine showed a decrease in complications and length of hospital stay (LOS) after surgery (18). In another study, no effect was seen after

abdominal surgery for cancer; however, this may be caused by an under-dosing treatment (0.2 g·kg^{-1} per day) (19). In patients with a risk of malnutrition before gastrointestinal surgery, supplementation of glutamine may shorten intensive care unit stay and improve insulin levels (20). In cardiac surgery, a perioperative high-dose glutamine administration (0.5 g·kg^{-1}·day^{-1}) did increase the glutathione concentration, and these increased glutathione levels remained after surgery (21). Glutathione is known to be protective against myocardial ischemia/reperfusion injury, which is associated with increased morbidity and mortality (22). Glutamine supplementation has a preserving effect on contractile function of cardiomyocytes after open-heart surgery (23,24). In patients undergoing gastrointestinal surgery, perioperative supplementation may be beneficial in ameliorating immune depression and shortening hospitalization (17,25).

Parenteral route: Postoperative supplementation. Characteristic features after surgical stress are hyperglycemia and cumulative nitrogen losses, which may increase the risk of infection, delay wound healing and diminish muscle strength after surgery, resulting in a prolonged hospital stay (26). This response can also be counteracted with postoperative parenteral glutamine supplementation. Glycemic control is associated with decreased total postoperative infections (27). Intravenous postoperative glutamine supplementation in surgical patients reduces infectious complication rates, shortens LOS and decreases hospital costs (28–31). The greatest benefit of intravenous supplementation was observed in patients receiving high-dose glutamine. Thus, a high degree of benefits is found in studies that used high doses of glutamine (30,31). The most optimal dose is probably 0.5 g·kg^{-1} per day (32).

In critically ill patients, low levels of glutamine also have been associated with immune dysfunction and higher mortality (33). Also, glutathione becomes depleted during critical illness and this is associated with a poor clinical outcome (34). In critically ill patients, intravenous administration of glutamine increased glutathione levels (35). Supplementation of parenteral glutamine in critically ill patients was associated with a reduction of urinary tract infections and nosocomial pneumonia (36).

Enteral route. Enteral supplementation has an advantage over parenteral supplementation. An early initiation of postoperative enteral nutrition shortens LOS, shows fewer complications and reduces infectious complications in patients undergoing major abdominal surgery compared with delayed enteral nutrition (37).

In trauma patients, supplementation of enteral glutamine lowered the incidence of pneumonia (38). In critically ill patients the addition of glutamine to enteral nutrition reduced LOS by more than 4 day (39). In trauma patients undergoing shock resuscitation, enteral glutamine administration was safe and enhances gastrointestinal tolerance (40). Postoperative ileus is a common complication after gastrointestinal surgery; however, glutamine acts as a motility-recovery agent (41). Not only is enteral glutamine protective via the enteral route for myocardial injury and clinical complications in patients undergoing cardiac surgery (42), it also has a protective effect on the epithelial barrier function. Enteral glutamine supplementation increases intestinal fractional extraction of glutamine. This higher intestinal fractional extraction is probably important to sustain physiological levels of glutathione and preserve heat shock protein 70 and it serves as a substrate to the gut-associated lymphoid tissue system.

Guidelines of professional nutrition societies currently recommend intravenous supplementation of glutamine in critically ill patients, as recent data support the use of glutamine in order to reduce mortality in these patients (43). Parenteral glutamine administration may also be beneficial in patients undergoing major surgery. The European Society for Parenteral and Enteral Nutrition guidelines state that 'some evidence exists' that intravenous glutamine administration to these patients can improve LOS and infection risk (44). These guidelines recommend the enteral route to deliver immune nutrients, but so far sufficient data are not available to support enteral glutamine supple- mentation in surgical patients in general (45). In a recent American Society for Parenteral and Enteral Nutrition (ASPEN) position paper on the use of parenteral glutamine supplementation, it is stated that it may be beneficial for certain adult surgical patients, for example, patients undergoing major abdominal surgery. However, the heterogeneity of the investigated patient populations makes this statement controversial. A growing body of data shows that glutamine supplementation with an optimal dose of 0.5 $g \cdot kg^{-1} \cdot day^{-1}$ may be beneficial for the recovery after surgery. The best results may be achieved by administering glutamine by both enteral and parenteral routes as soon as possible after surgery (46). However, caution is advised in patients with renal failure and severe hepatic dysfunction, since studies suggest that glutamine may be harmful and more evidence for this patient population is needed (47,48). Further high-quality research is necessary to confirm the afore-mentioned perspectives. The results of the REDOXS trial will be available soon, which may give more insight into the role of glutamine in clinical care (49). (The REDOX study will be discussed in Chapter Five of this thesis)

Arginine

Background

Arginine is a conditionally essential amino acid with several pharmacological properties, which becomes depleted during stress associated with surgery and trauma. Arginine is an immune enhancing nutrient, because it is essential for an adequate immune response, since it is the substrate for normal T-lymphocyte development (50). T-lymphocytes depend on adequate arginine levels for proliferation, the expression of the T-lymphocyte receptor complex and the z-chain peptide, and the development of immunological memory (51). Furthermore, arginine is the sole precursor for NO. This versatile substance has cytotoxic properties to kill parasites, bacteria and viruses. It has an important signaling role for immune cells by regulating cytokine activation and receptor presentation, and it is the regulator of organ perfusion. In addition, arginine improves the process of wound healing (52). Because of all these properties, arginine is often called an immune nutrient.

Patients undergoing surgical injury develop an arginine deficiency and consequently an impaired immune function (53). Since arginine levels drop ± 50 % within a few hours after surgery, it is suggested that arginine deficiency is caused not by decreased intake, but rather through a disturbance in arginine metabolism (53). Arginine is mainly catabolized by two competing enzymes: inducible nitric oxide synthase (iNOS) and arginase. iNOS metabolizes arginine into NO. Arginine availability is the regulating factor of NO production. Arginase, which converts arginine into urea and ornithine, is the only enzyme that is really capable of decreasing arginine levels and thus NO production.

After traumatic injury, for example surgery, immature cells of myeloid origin are found in the circulation, lymph nodes, liver and spleen. These so-called myeloid-derived suppressor cells (MDSC) express the enzymes iNOS and arginase (54). The expression of both enzymes is regulated by cytokines of T-helper (Th) cells. Th1 cytokines are pro-inflammatory and promote iNOS expression; Th2 cytokines are anti-inflammatory and induce arginase expression (55). In physiological conditions this regulation is in balance; however, in patients with injury the balance is disturbed. Surgical stress causes a predominant production of Th2 cytokines and this promotes the MDSC to express arginase (56,57). Thus, after surgery, arginase-producing MDSC appear and cause an arginine deficiency. Consequently, NO metabolites are decreased in patients with physical injury

because of a perturbation in NO production (58). This results in the suppression of the T-lymphocyte dependent immune function and NO activity and this is a plausible explanation for the impaired immune function after surgery.

The described mechanism suggests that physical injury caused by surgery induces an arginine deficiency, which can be restored with arginine supplementation. Experimental studies have shown that arginine administration improves wound healing, restores macrophage and T-lymphocyte function and augments resistance to infectious pathogens (50,52). Furthermore, arginine supplementation increases NO and improves microcirculation after injury (59). Other studies have shown that preoperative arginine-enriched nutrition improves immune function and decreased the production of Th2 cytokines (60,61). Several clinical studies have shown that a correction of the arginine deficiency by arginine-enriched nutrition restores T-lymphocyte count and function in surgical patients (50,62).

Other promising ingredients in immune nutrition are *n*-3 PUFA, which are often administered in combination with arginine. *n*-3 PUFA also interfere with arginine metabolism by decreasing Th2 cytokines and thereby maintaining the Th1/Th2 balance. This results in a decrease in arginase activity and inhibits arginine breakdown (63). The role of *n*-3 PUFA in surgery will be outlined in more detail.

Arginine supplementation in surgical patients

Adequate clinical data on the effects of parenteral arginine supplementation in surgical patients are lacking. Nevertheless, in the past 20 years many randomized clinical trials have been performed to examine the effects of arginine-enriched enteral nutrition in various settings and nutrition compositions. Six major meta-analyses reviewing these trials in surgical and trauma patients have been published (64–69). The two most recent studies by Marik et al. and Drover et al. describe both substantial reduction in postoperative complications and a shorter LOS with the use of arginine administration. They found no overall effect on mortality compared with standard perioperative nutritional care. Pre-, peri- and postoperative administration of arginine-enriched nutrition is associated with a reduction of postoperative complications, and both perioperative and postoperative use of arginine supplementation were associated with a reduction in LOS. A greater effect of arginine is assumed when it is administered in both the pre- and postoperative phases. However, there exists considerable heterogeneity in the different trials examining the effects of arginine-enriched diets, likely due to differences in patients, local practice protocols, health care systems, study designs,

diet compositions and other methodologies. Furthermore, there are only a few studies using arginine as a sole pharmaco-nutrient in the intervention group. In most studies, the immune-enhancing diet consisted of arginine in combination with glutamine, *n*-3 PUFA and antioxidants, which makes it hard to ascribe the effects to a sole nutritional substrate. The variety in study methodology may also be ascribed to the wide time span in which the trials are performed, because the results of later studies might be influenced by new treatment opportunities.

The use of arginine-enriched nutrition in oncology deserves special attention. Almost all clinical trials mentioned earlier included patients who underwent curative oncological surgery. A malignant tumor also disturbs the arginine metabolism of the host (70). The initial concept is quite similar to the alterations seen after surgery. Cancer by itself recruits MDSC from the moment of carcinogenic initiation (71). During the first phases of carcinogenesis the tumor-derived MDSC seem to produce arginase to pre- vent the immune system from fighting the malignant cells. However, during outgrow of the malignant tumor, the Th1/Th2 balance switches to an increased Th1 cytokine production in the tumor environment, which promotes the MDSC to activate high amounts of iNOS (72). In this stage, arginine is converted into NO by iNOS. This results in pathologically high NO levels, promoting angiogenesis and microcirculation in the tumor environment. Furthermore, in the presence of increased iNOS activity and low arginine levels, radical nitrogen species will be formed, which damage the surrounding cells even more. This might explain the controversial outcomes of studies in patients with inoperable advanced metastatic cancer. Arginine supplementation in this advanced metastatic phase may even worsen clinical outcome. This is supported by studies on the effects of supplemental arginine in critically ill patients with sepsis. In sepsis, the Th1/Th2 balance is also shifted to the Th1 side and extra exogenous arginine in septic patients causes no benefit, and perhaps even harm (73).

However, it is hypothesized that the pronounced positive effects of perioperative arginine supplementation may be explained by the return of the Th1/Th2 balance (and therefore the iNOS/arginase balance) to the Th2 side after surgery (74,75).

The guidelines from leading nutrition societies in the world recommend the use of immune enhancing arginine enriched nutrition in perioperative care of patients undergoing major abdominal surgery, head and neck surgery and after severe trauma, with caution in patients with severe sepsis (76,77). Perioperative arginine supplementation in patients with a malignancy of the digestive tract may

be beneficial (78); however, arginine administration to patients with progressive non-curable cancer has to be avoided. Bozzetti has stated that immune enhancing diets containing arginine are preferable to the standard enteral formulae in the preoperative setting (79). It can be concluded that arginine supplemented enteral diets should be prescribed to all patients undergoing elective surgery.

n-3 PUFA

Inflammation is a common sequel to surgery. The regulation of inflammation depends on a balance between pro- and anti-inflammatory mediators. When regulated adequately, inflammation is essential for recovery after surgical injury. However, when the balance is disturbed, this intentional protection mechanism becomes damaging for the host (80). Pathological inflammation is a result of this disturbance and may evolve into severe complications, for example, sepsis, multi-organ failure or acute respiratory distress syndrome (32). The pharmaco-nutrients *n*-3 PUFA have anti-inflammatory properties and may overcome this postoperative morbidity by restoring the balance between pro- and anti-inflammatory mediators.

Background

n-3 PUFA from fish oil may impair inflammatory responses (81). Eicosanoids and leukotriene mediators are signaling molecules with an important regulatory function in the inflammatory response. These signaling mediators are the products of either *n*-3 PUFA or *n*-6 PUFA. In general, the *n*-6 PUFA are the precursors for pro-inflammatory mediators and the *n*-3 PUFA are metabolized into less inflammatory mediators (82). The *n*-3/*n*-6 PUFA balance in the membranes of inflammatory cells, for example, neutrophils and macrophages, regulates the inflammatory response. In this way, *n*-3 PUFA have anti-inflammatory actions, as substitutes for *n*-6 PUFA in the cell membranes of inflammatory cells and thereby diminish pro-inflammatory mediator production. Furthermore, *n*-3 PUFA block the production of *n*-6 PUFA-derived mediators by competing for the metabolic enzymes necessary for the conversion into the pro- inflammatory mediators (83). In addition, another anti-inflammatory effect of *n*-3 PUFA is caused by their role as precursors for resolvins and protectins. These resolvins and protectins have multiple anti-inflammatory properties, for example, inhibition of accumulation of dendritic cells and neutrophils, stimulation of macrophages and decreasing the production of pro-inflammatory cytokines (84). The inflammatory condition

or even the systemic inflammatory response syndrome seen after surgery may be a result of a misbalance between *n*-3 PUFA and *n*-6 PUFA. As a result of the high intake of *n*-6 PUFA and the low intake of *n*-3 PUFA, cell membranes of Western populations are dominated by *n*-6 PUFA. Adequate supplementation of *n*-3 PUFA may restore the membrane composition and thereby resolve the regulation of the inflammation response and promote recovery after surgery (85).

n-3 PUFA supplementation in surgical patients

Supplementation of *n*-3 PUFA is expected to have beneficial effects in inflammatory circumstances, such as surgery and systemic inflammatory response syndrome. Three recent systematic reviews outline the effects of the supplementation of *n*-3 PUFA and two of them focus on parenteral supplementation (86–88).

Parenteral route. Based on a meta-analysis, it may be presumed that parenteral supplementation of *n*-3 PUFA in patients undergoing major surgery is not only safe, but may also decrease the risk of postoperative infections and reduce LOS (86,88). Van der Meij et al. evaluated the effects of *n*-3 PUFA in both general surgery and oncological surgery separately. This qualitative review did not find any effects of perioperative *n*-3 PUFA supplementation on infection rate and mortality in surgical patients. In patients undergoing surgery for a malignancy receiving parenteral *n*-3 PUFA, LOS was shorter. In patients without cancer, the effects of parenteral *n*-3 PUFA supplementation on LOS were inconsistent. Although the studies did not report a significant improvement in mortality rate in patients receiving parenteral *n*-3 PUFA, a trend towards a decrease in hospital costs was observed compared with control groups (89). A recently published study on the effect of post- operative parenteral *n*-3 PUFA supplementation in surgical critically ill patients showed a significant decrease in the hyperinflammatory response after major surgery, a reduction in the production of pro-inflammatory cytokines and a tendency for less postoperative infections in the intervention group (85). In most studies, the parenteral solution with *n*-3 PUFA was administered in the post-operative period. Only a few studies combined postoperative and preoperative administration of *n*-3 PUFA (90,91), and meaningful conclusions on the ideal administration period of *n*-3 PUFA cannot be drawn from these studies. However, parenteral administration of *n*-3 PUFA down-regulated the *n*-6/*n*-3 ratio in plasma and cell membrane in a relatively short time span (1–3 days) (87). This suggests that the highest treatment effect can be reached by starting the administration of parenteral *n*-3 PUFA a few days before surgery.

Enteral route. The systematic review of van der Meij et al. found only three randomized controlled trials of acceptable quality looking into the effects of enteral nutrition enriched with *n*-3 PUFA in surgical oncology (87). No studies investigated the effects of these nutrients on general non-cancer surgery. Overall, these studies did not provide evidence for clinical benefits of postoperative enteral supplementation of *n*-3 PUFA. However, a tendency for fewer infectious complications in surgical patients who received an enteral formula with *n*-3 PUFA for 7d postoperatively was reported (92,93). In a recently published study of high quality in patients undergoing oesophagastric cancer surgery, perioperative *n*-3 PUFA supplementation did not affect the immune function and clinical outcome (94). However, one study showed preservation of the body weight and lean body mass, whereas both decreased in the control group (95). Basal research in healthy volunteers shows that the incorporation of *n*-3 PUFA after enteral supplementation occurred after approximately 4–7 days and reaches a new steady state composition within approximately 4 weeks in a dose-response fashion (96). Clinical studies examining the effects of enteral nutrition containing high amounts of *n*-3 PUFA as well as γ-linolenic acid and antioxidants, consistently showed significant clinical benefits in patients with other inflammatory diseases, for example, acute respiratory distress syndrome or sepsis (32,97,98).

The supplementation of *n*-3 PUFA is widely investigated in studies using commercially available enteral immune enhancing formulae, containing *n*-3 PUFA in combination with arginine, antioxidants and other immune modulating nutrients. Although these studies report many beneficial clinical effects of these immune enhancing formulae and international guidelines recommend the administration of this nutrition in patients undergoing major surgery, interpretation of the data in this area is difficult due to various amounts of *n*-3 PUFA present in the different enteral formulations and the inclusion of other immune modulating nutrients in the formulae (65,82).

From the available clinical data, it can be concluded that there is insufficient evidence to recommend the oral or enteral supplementation of *n*-3 PUFA in oncological or general patients undergoing surgery. However, *n*-3 PUFA might improve inflammatory response after surgery relying on its potential anti-inflammatory properties. In patients with acute respiratory distress syndrome and sepsis, the administration of enteral nutrition containing *n*-3 PUFA is recommended. Parenteral supplementation of *n*-3 PUFA-enriched formulae might be considered in the perioperative period (e.g. during postoperative recovery or complications such as acute respiratory distress syndrome or sepsis).

Taurine

Taurine is a nutrient with regulating properties in both the immune system and energy supply. Clinical data on the effect of taurine supplementation in surgical patients are lacking, but the potential of this pharmaco-nutrient in perioperative care will be outlined.

Taurine is a semi-essential aminosulfonic acid and its sulfonate group makes taurine highly acidic, which makes it a zwitterion. As a zwitterion, taurine is able to function as a buffer when pH is low and function as a hydrogen ion donor when pH is high. Thus, taurine is very important in maintaining the acid–base homoeostasis in the body. A disturbance in this homoeostasis may be induced by surgery and associated factors, for example, mechanical ventilation, medication, the stress response and alterations in the fluid compartments of the body during surgery.

Taurine is an osmolyte that controls fluid movement and ion fluxes across cell membranes (99). Surgery causes oxidative stress in several organs, for example, through ischemia/reperfusion injury, which exerts an osmotic imbalance. This may be reflected as postoperative edema: an excessive shift from body fluids to the intracellular space. However, when taurine is released from the swollen cells, ions and water will move from the intracellular space to the extracellular space, suggesting that edema occurs when taurine is conditionally essential. In this way, taurine might be a potential protector against surgery-induced oxidative damage.

Furthermore, other experimental data show that taurine plays a role in the inflammation response and immune system. Taurine has been shown to down-regulate pro-inflammatory cytokines and function as an antioxidant at the site of inflammation (100,101). Moreover, taurine uptake by T-cells is crucial for the survival and the immune reactions of these cells and a decrease in taurine uptake results in a reduction of T-cell responses (102).

In response to surgical injury, plasma taurine levels decrease, which suggests an increased metabolic requirement (103). Substantial evidence for the effects of taurine supplementation in surgical patients is absent and further studies are needed. However, with no known harmful effects and with much evidence suggesting a potential role for taurine in the recovery from surgical injury and inflammation, taurine supplementation may have positive effects.

Carbohydrates

For some years, guidelines have stated that preoperative fasting is an unwanted phenomenon (104). However, fasting before surgery is still common practice in preoperative care in many Western countries (105).

Background

Fasting for 8 hours before surgery results in depletion of glycogen stores in the liver. Subsequently, glucose has to be released in alternative ways, mainly by the mobilization of glycogen from the muscle by eliciting a stress response. This response has consequences for the physical condition of the patient, because levels of cortisol, adrenaline and other signaling mediators are elevated. This interplay may result in insulin resistance at the level of the liver and muscle. Moreover, energy stores are depleted in the gastrointestinal tract, liver, kidneys, heart and lungs. Insulin resistance is not a favorable state of the body, because it may lead to increased infectious complications and prolonged hospital stay.

Preoperative carbohydrate loading

To avoid this unwanted stress response, patients can be given a sufficient amount of CHO, via the intravenous route or via the enteral route shortly (2–3 hours) before surgery. CHO loading preserves the energy status of the liver and most importantly reduces insulin resistance (106). Also, it improves intestinal integrity and reduces bacterial trans- location (107).

Parenteral route. Clinical studies in patients showed that intravenous CHO supplementation in sufficient amounts reduces the postoperative infection rate and improves wound healing (108,109). In patients undergoing cardiac surgery, intravenous CHO loading is effective in overcoming the fasted state and this results in less myocardial damage (110). Although intravenous CHO loading has proved to be successful in overcoming a fasted state and in exhibiting beneficial effects, this way of administration has certain disadvantages. For instance, high dosages (5 $mg \cdot kg^{-1} \cdot min^{-1}$ or more) are needed to counteract the insulin resistance (111). Also, intravenous administration of glucose requires concomitant insulin infusion, which needs frequent monitoring of blood glucose levels and the risk of fluid overload.

Enteral/Oral route. An easier way to reach an optimal metabolic effect is by giving an oral CHO drink (105). To attain beneficial effects in a clinical setting, the drink must contain at least 48 g CHO; which is the amount needed to overcome the fasted state and change it to a fed state. Up to 2 hours before surgery an iso-osmolar CHO drink has proven to be safe in patients. After ingestion, the stomach empties the CHO drink within 90min, thereby not increasing the risk of gastric aspiration during anesthesia (112). Preoperative supplementation of CHO in amounts of 800 ml during the evening before the operation and 400 ml 2–3 hours before the operation was investigated extensively. Regarding clinical parameters, a reduction in preoperative discomfort (e.g. feeling of thirst and hunger), postoperative nausea and vomiting, and a shorter LOS were demonstrated in prospective, randomized trials (113–117). Also, the unwanted insulin resistance after surgery was shown to be reduced (117,118). Other studies demonstrated an earlier return of gastrointestinal function and a preserved muscle mass and strength (116,119). Recently, a study demonstrated that preoperative CHO loading causes less immune suppression in terms of the human leucocyte antigen HLA-DR expression in monocytes (115).

Preoperative CHO loading has many positive clinical effects and no disadvantages have been reported. However, outcome measures such as morbidity and mortality have not yet been explored. Also, the effects of CHO loading in populations with a proposed altered CHO metabolism, such as obese or overweight patients, have not been investigated. It may be concluded that a simple intervention with a preoperative CHO supplementation may contribute to the well-being of the patient and that in this perspective preoperative fasting is outdated.

Summary

In summary, surgical injury causes various changes in the immune function and the body's homeostasis. This review outlines the potential role of several pharmaco-nutrients in perioperative care, to improve recovery (Table 1). The combination of both parenteral and enteral glutamine supplementation might improve postoperative outcome; however, the results of large randomized trials of high quality are awaited. Supplementation of immune enhancing formulae with arginine and *n*-3 PUFA in the perioperative setting has been shown to be beneficial, with special attention to surgical oncology. Although data are limited, taurine has the potential to improve the physical condition of the surgical patient.

Besides the specialized nutrients, adequate CHO intake before surgery should now be common practice.

It is important to realize that a relatively simple intervention with these pharmaconutrients may improve the postoperative recovery of surgical patients. Nutritional interventions should gain more ground in perioperative care.

TABLE 1 | Summary of recommendations on substrates in surgery

Substrate	Patients	Application
Glutamine	Surgical ICU patients Burn and trauma patients	Preferable in EN, glutamine dipeptide Start EN in the ICU setting When PN is indicated, add glutamine dipeptide 0.3-0.5 $g \cdot kg^{-1} \cdot day^{-1}$ glutamine dipeptide
Arginine	Major abdominal surgery Head and Neck surgery Severe trauma	Preferable in immune enhancing EN Start 7-5 days before surgery Until 10 days after surgery
Omega-3 PUFA	Major abdominal surgery Head and Neck surgery Severe trauma	Preferable in immune enhancing EN Start 7-5 days before surgery Until 10 days after surgery
Taurine	(limiting data)	(limiting data)
CHO loading	All elective surgery	CHO-rich drink before surgery 800 ml (100mg) and 400 ml (50 g) CHO 8 and 2 hours preoperatively respectively

This table shows a summary of the authors recommendations on the use of the nutritional substrates in surgery. On the date of publication, ESPEN, ASPEN and ASA guidelines are in line with these statements (77,78,107). ICU, intensive care unit; EN, enteral nutrition; PN, parenteral nutrition; CHO, carbohydrates.

References

1. Coeffier M, Marion R, Ducrotte P et al. Modulating effect of glutamine on IL-1beta-induced cytokine produc- tion by human gut. Clin Nutr 2003;22:407–413.
2. Melis GC, ter Wendel N, Boelens PG, van Leeuwen PAM. Glutamine: recent developments in research on the clinical significance of glutamine. Curr Opin Clin Nutr Metab Care 2004;7:59–70.
3. Juretic A, Spagnoli GC, Horig H et al. Glutamine requirements in the generation of lymphokine-activated killer cells. Clin Nutr 1994;13:42–49.
4. Vermeulen MA, van de Poll MC, Ligthart-Melis GC, et al. Specific amino acids in the critically ill patient–exogenous glutamine/arginine: a common de-nominator? Crit Care Med 2007;35:S568–S576.
5. Flaring UB, Rooyackers OE, Wernerman J et al. Glutamine attenuates post-traumatic glutathione depletion in human muscle. Clin Sci (Lond) 2003;104:275–282.
6. Singleton KD, Serkova N, Beckey VE et al. Glutamine attenuates lung injury and improves survival after sepsis: role of enhanced heat shock protein expression. Crit Care Med 2005;33:1206–1213.
7. Ziegler TR, Ogden LG, Singleton KD et al. Parenteral glutamine increases serum heat shock protein 70 in critically ill patients. Intensive Care Med 2005;31:1079– 1086.
8. Li N, Neu J. Glutamine deprivation alters intestinal tight junctions via a PI3-K/Akt mediated pathway in Caco-2 cells. J Nutr 2009;139:170–714.
9. van de Poll MC, Ligthart-Melis GC, Boelens PG et al. Intestinal and hepatic metabolism of glutamine and citrulline in humans. J Physiol 2007;581:819–827.
10. van de Poll MC, Siroen MP, van Leeuwen PA et al. Interorgan amino acid exchange in humans: consequences for arginine and citrulline metabolism. Am J Clin Nutr 2007;85:167–172.
11. Melis GC, Boelens PG, van der Sijp JR et al. The feeding route (enteral or parenteral) affects the plasma response of the dipetide Ala-Gln and the amino acids glu- tamine, citrulline and arginine, with the administration of Ala-Gln in preoperative patients. Br J Nutr 2005;94:19–26.
12. Boelens PG, Houdijk AP, de Thouars HN, et al. Plasma taurine concentrations increase after enteral glutamine supplementation in trauma patients and stressed rats. J Nutr 2003;131:2569S–2577S.
13. Nose K, Yang H, Sun X, et al. Glutamine prevents total parenteral nutrition-associated changes to intraepithelial lymphocyte phenotype and function: a potential mechanism for the preservation of epithelial barrier function. J Interferon Cytokine Res 2010;30:678–680.
14. Ligthart-Melis GC, van de Poll MC, Dejong CH et al. The route of administration (enteral or parenteral) affects the conversion of isotopically labeled L-[2– 529. 15N]glutamine into citrulline and arginine in humans. JPEN J Parenter Enteral Nutr 2007;31:343–348.
15. Asprer JM, Llido LO, Sinamban R et al. Effect on immune indices of preoperative intravenous glutamine dipeptide supplementation in malnourished abdominal surgery patients in the preoperative and postoperative periods. Nutrition 2009;25:920–925.

16. Exner R, Tamandl D, Goetzinger P et al. Perioperative GLY-GLN infusion diminishes the surgery-induced period of immunosuppression: accelerated restoration of the lipopolysaccharide-stimulated tumor necrosis factor-alpha response. Ann Surg 2003;237:110–115.
17. Yao GX, Xue XB, Jiang ZM et al. Effects of peri- operative parenteral glutamine-dipeptide supplementation on plasma endotoxin level, plasma endotoxin inactivation capacity and clinical outcome. Clin Nutr 2005;24:510–515.
18. Oguz M, Kerem M, Bedirli A et al. L-alanine–L- glutamine supplementation improves the outcome after colorectal surgery for cancer. Colorectal Dis 2007;9;515–520.
19. Jo S, Choi SH, Heo JS. Missing effect of glutamine supplementation on the surgical outcome after pancreaticoduodenectomy for periampullary tumors: a prospective, randomized, double- blind, controlled clinical trial. World J Surg 2006;30:1974–1982.
20. Mercadal OG, Llop Talaveron JM. Effectiveness of perioperative glutamine in parenteral nutrition in patients at risk of moderate to severe malnutrition. Nutr Hosp 2011;26:1305–1312.
21. Engel JM, Muhling J, Kwapisz M et al. Glutamine administration in patients undergoing cardiac surgery and the influence on blood glutathione levels. Acta Anaesthesiol Scand 2009;53:1317–1323.
22. Domanski MJ, Mahaffey K, Hasselblad V et al. Association of myocardial enzyme elevation and survival following coronary artery bypass graft surgery. JAMA 2011;305:585–591.
23. Lomivorotov VV, Efremov SM, Shmirev VA et al. Glutamine is cardioprotective in patients with ischemic heart disease following cardiopulmonary bypass. Heart Surg Forum 2011;14:E384–E388.
24. Wischmeyer PE, Vanden Hoek TL, Li C et al. Glutamine preserves cardiomyocyte viability and enhances recovery of contractile function after ischemia-reperfusion injury. JPEN J Parenter Enteral Nutr 2003;27:116–122.
25. Yeh CN, Lee HL, Liu YY et al. The role of parenteral glutamine supplement for surgical patient perio- peratively: result of a single center, prospective and controlled study. Langenbecks Arch Surg 2008;393:849–855.
26. Schricker T, Lattermann R. Strategies to attenuate the catabolic response to surgery and improve perioperative outcomes. Can J Anaesth 2007;54:414–419.
27. Fukushima R, Inaba T, Iinuma H et al. Periopera- tive nosocomial infection preventive measures. Nihon Geka Gakkai Zasshi 2004;105:696–701.
28. Avenell A. Glutamine in critical care: current evidence from systematic reviews. Proc Nutr Soc 2006;65:236–241.
29. Estivariz CF, Griffith DP, Luo M et al. Efficacy of parenteral nutrition supplemented with glutamine dipeptide to decrease hospital infections in critically ill surgical patients. JPEN J Parenter Enteral Nutr 2008;32:389–402.
30. Novak F, Heyland DK, Avenell A et al. Glutamine supplementation in serious illness: a systematic review ofthe evidence. Crit Care Med 2002;30:2022–2029.
31. Wang Y, Jiang ZM, Nolan MT et al. The impact of glutamine dipeptide-supplemented parenteral nutrition on outcomes of surgical patients: a meta-analysis of randomized clinical trials. JPEN J Parenter Enteral Nutr 2010;34:521–524.
32. Wischmeyer P. Nutritional pharmacology in surgery and critical care: 'you must unlearn what you have learned'. Curr Opin Anaesthesiol 2011;24:381–388.

33. Oudemans-van Straaten HM, Bosman RJ, Treskes M et al. Plasma glutamine depletion and patient outcome in acute ICU admissions. Intensive Care Med 2001;27:84–90.
34. Flaring UB, Rooyackers OE, Hebert C, Bratel T, Hammarqvist F, Wernerman J. Temporal changes in whole-blood and plasma glutathione in ICU patients with multiple organ failure. Intensive Care Med 2005;31:1072–1078.
35. Eroglu A. The effect of intravenous alanyl-glutamine supplementation on plasma glutathione levels in intensive care unit trauma patients receiving enteral nutrition: the results of a randomized controlled trial. Anesth Analg 2009;109:502–505.
36. Grau T, Bonet A, Minambres E et al. The effect of L-alanyl–L-glutamine dipeptide supplemented total parenteral nutrition on infectious morbidity and insulin sensitivity in critically ill patients. Crit Care Med 2001;39:1263–1268.
37. Marik PE, Zaloga GP. Early enteral nutrition in acutely ill patients: a systematic review. Crit Care Med 2001;29:2264–2270.
38. Houdijk AP, Rijnsburger ER, Jansen J et al. Randomised trial of glutamine-enriched enteral nutrition on infectious morbidity in patients with multiple trauma. Lancet 1998;352:772–776.
39. McClave SA, Heyland DK. The physiologic response and associated clinical benefits from provision of early enteral nutrition. Nutr Clin Pract 2009;24:305–315.
40. McQuiggan M, Kozar R, Sailors RM et al. Enteral glutamine during active shock resuscitation is safe and enhances tolerance of enteral feeding. JPEN J Parenter Enteral Nutr 2008;32:28–35.
41. Mochiki E, Ohno T, Yanai M et al. Effects of glutamine on gastrointestinal motor activity in patients following gastric surgery. World J Surg 2011;35:805–810.
42. Sufit A, Weitzel LB, Hamiel C et al. Pharmacologically dosed oral glutamine reduces myocardial injury in patients undergoing cardiac surgery: a randomized pilot feasibility trial. JPEN J Parenter Enteral Nutr 2012;36:556–561.
43. Heyland DK. Clinical Practice Guidelines 2009. http:// www.criticalcarenutrition.com
44. Braga M, Ljungqvist O, Soeters P et al. ESPEN guidelines on parenteral nutrition: surgery. Clin Nutr 2009;28:378–386.
45. Schulman AS, Willcutts KF, Claridge JA et al. Does the addition of glutamine to enteral feeds affect patient mortality? Crit Care Med 2005;33:2501–2506.
46. Kim M, Wischmeyer PE. Glutamine. World Rev Nutr Diet 2013;105:90–96.
47. Hubl W, Druml W, Roth E, Lochs H. Importance of liver and kidney for the utilization of glutamine-containing dipeptides in man. Metabolism 1994;43:1104–1107.
48. Rama Rao KV, Yayakumar AR, Norenberg MD. Glutamine in the pathogenesis of acute hepatic encephalopathy. Neurochem Int 2012;61:575–580.
49. Heyland DK, Dhaliwalm R, Day A et al. Optimizing the dose of glutamine dipeptides and antioxidants in critically ill patients: a phase I dose-finding study. JPEN J Parenter Enteral Nutr 2007;31:109–118.
50. Popovic PJ. Arginine and immunity. J Nutr 2007;137:1681S–1686S.
51. Ochoa JB, Strange J, Kearney P et al. Effects of L-arginine on the proliferation of T lymphocyte sub-populations. JPEN J Parenter Enteral Nutr 2001;25:23–29.
52. Witte MB, Barbul A. Arginine physiology and its implication for wound healing. Wound Repair Regen 2003;11:419–423.
53. Zhu X, Herrera G, Ochoa JB. Immunosupression and infection after major surgery: a nutritional deficiency. Crit Care Clin 2010;26:491–500, ix.

54. Makarenkova VP, Bansal V, Matta BM et al. CD11b + /Gr-1 + myeloid suppressor cells cause T cell dysfunction after traumatic stress. J Immunol 2006;176;2085–2094.

55. Holan V, Pindjakova J, Krulova M et al. Production of nitric oxide during graft rejection is regulated by the Th1/Th2 balance, the arginase activity, and L-arginine metabolism. Transplantation 2006;81:1708–1715.

56. Chiarla C, Giovannini I, Siegel JH. Plasma arginine correlations in trauma and sepsis. Amino Acids 2006;30:81–86.

57. Bansal V, Ochoa JB. Arginine availability, arginase, and the immune response. Curr Opin Clin Nutr Metab Care 2003;6:223–228.

58. Jacob TD, Ochoa JB, Udekwu AO et al. Nitric oxide production is inhibited in trauma patients. J Trauma 1993;35:590–596.

59. Krauss H, Jablecka A, Sosnowski P et al. Influence of L-arginine on the nitric oxide concentration and level of oxidative stress during ischemia-reperfusion injury in a rat model. Int J Clin Pharmacol Ther 2009;47:533–538.

60. Matsuda A, Furukawa K, Takasaki H et al. Pre- operative oral immune-enhancing nutritional supplementation corrects TH1/TH2 imbalance in patients undergoing elective surgery for colorectal cancer. Dis Colon Rectum 2006;49:507–516.

61. Tepaske R, Velthuis H, Oudemans-van Straaten HM et al. Effect of preoperative oral immune-enhancing nutritional supplement on patients at high risk of infection after cardiac surgery: a randomised placebo-controlled trial. Lancet 2001;358:696–701.

62. Braga M, Gianotti L, Vignali A et al. (2002) Preoperative oral arginine and n-3 fatty acid supplementation improves the immunometabolic host response and outcome after colorectal resection for cancer. Surgery 2002;132:805–814.

63. Marik PE, Flemmer M. Immunonutrition in the surgical patient. Minerva Anestesiol 2012;78:336–342.

64. Drover JW, Dhaliwal R, Weitzel L et al. Perioperative use of arginine-supplemented diets: a systematic review of the evidence. J Am Coll Surg 2011;212:385–399.

65. Marik PE, Zaloga GP. Immunonutrition in high- risk surgical patients: a systematic review and analysis of the literature. JPEN J Parenter Enteral Nutr 20120;34:378–386.

66. Montejo JC, Zarazaga A, Lopez-Martinez J et al. Immunonutrition in the intensive care unit. A systematic review and consensus statement. Clin Nutr 2003;22:221–233.

67. Heyland DK, Novak F, Drover JW et al. (2001) Should immunonutrition become routine in critically ill patients? A systematic review of the evidence. JAMA 2001;286:944–953.

68. Beale RJ, Bryg DJ, Bihari DJ (1999) Immunonutrition in the critically ill: a systematic review of clinical outcome. Crit Care Med 1999;27:2799–2805.

69. Heys SD, Walker LG, Smith I et al. Enteral nutritional supplementation with key nutrients in patients with critical illness and cancer: a meta-analysis of randomized controlled clinical trials. Ann Surg 1999;229:467–477.

70. Vissers YL, Dejong CH, Luiking YC, Fearon KC, von Meyenfeldt MF, Deutz NE. Plasma arginine concentrations are reduced in cancer patients: evidence for arginine deficiency? Am J Clin Nutr 2005;81:1142–1146.

71. Rodriguez PC, Ochoa AC. Arginine regulation by myeloid derived suppressor cells and tolerance in cancer: mechan- isms and therapeutic perspectives. Immunol Rev 2008;222:180–191.

72. Redente EF, Orlicky DC, Bouchard RJ, Malkinson AM. Tumor signaling to the bone marrow changes the phenotype of monocytes and pulmonary macrophages during urethane-induced primary lung tumorigenesis in A/J mice. Am J Pathol 2007;170:693–708.
73. Heyland DK, Samis A. Does immunonutrition in patients with sepsis do more harm than good? Intensive Care Med 2003;29:669–671.
74. Braga M, Gianotti L, Radaelli G et al. Perioperative immunonutrition in patients undergoing cancer surgery: results of a randomized double-blind phase 3 trial. Arch Surg 1999;134:428–433.
75. Buijs N, van Bokhorst-de van der Schueren MA, Langius JA et al. Perioperative arginine-supplemented nutrition in malnourished patients with head and neck cancer improves long-term survival. Am J Clin Nutr 2010;92:1151– 1156.
76. McClave SA, Martindale RG, Vanik VW et al. Guidelines for the provision and assessment of nutrition support therapy in the adult critically ill patient: Society of Critical Care Medicine (SCCM) and American Society for Parenteral and Enteral Nutrition (A.S.P.E.N.). JPEN J Parenter Enteral Nutr 2009;33:277–316.
77. Weimann A, Braga M, Harsanyi L et al. ESPEN guidelines on enteral nutrition: surgery including organ transplantation. Clin Nutr 2006;25:224– 244.
78. Paccagnella A, Morassutti I, Rosti G. Nutritional intervention for improving treatment tolerance in cancer patients. Curr Opin Oncol 2011;23:322–330.
79. Bozzetti F. Peri-operative nutritional management. Proc Nutr Soc 2011;70(3):305–310.
80. Calder PC. Omega-3 fatty acids and inflammatory processes. Nutrients 2010;2(3):355–374.
81. Santora R, Kozar RA. Molecular mechanisms of pharmaconutrients. J Surg Res 2010;161:288–294.
82. Stapleton RD, Martin JM, Mayer K. Fish oil in critical illness: mechanisms and clinical applications. Crit Care Clin 2010;26:501–14, ix.
83. Cahill NE, Dhaliwal R, Day AG et al. Nutrition therapy in the critical care setting: what is 'best achievable' practice? An international multicenter observational study. Crit Care Med 2010;38:395–401.
84. Stables MJ, Gilroy DW. Old and new generation lipid mediators in acute inflammation and resolution. Prog Lipid Res 2011;50:35–51.
85. Han YY, Lai SL, Ko WJ et al. Effects of fish oil on inflammatory modulation in surgical intensive care unit patients. Nutr Clin Pract 2012;27:91–98.
86. Chen B, Zhou Y, Yang P et al. Safety and efficacy of fish oil-enriched parenteral nutrition regimen on post- operative patients undergoing major abdominal surgery: a meta-analysis of randomized controlled trials. JPEN J Parenter Enteral Nutr 2010;34:387–394.
87. van der Meij BS, van Bokhorst-de van der Schueren MA, Langius JA et al. n-3 PUFAs in cancer, surgery, and critical care: a systematic review on clinical effects, incorporation, and washout of oral or enteral compared with parenteral supplementation. Am J Clin Nutr 2011;94:1248–1265.
88. Wei C, Hua J, Bin C et al. Impact of lipid emulsion containing fish oil on outcomes of surgical patients: systematic review of randomized controlled trials from Europe and Asia. Nutrition 2010;26:474–481.
89. Gao J, Ji CY, Wu GH. Use of fish oil lipid emulsion in patients undergoing major surgery and those with systemic inflammatory response syndrome: a cost-effectiveness analysis. Zhonghua Wei Chang Wai Ke Za Zhi 2012;15:452–456.

90. Heidt MC, Vician M, Stracke SK et al. Beneficial effects of intravenously administered N-3 fatty acids for the prevention of atrial fibrillation after coronary artery bypass surgery: a prospective randomized study. Thorac Cardiovasc Surg 2009;57:276–280.

91. Weiss G, Meyer F, Matthies B et al. Immunomodulation by perioperative administration of *n*-3 fatty acids. Br J Nutr 2002;87(Suppl. 1);S89–S94.

92. Kenler AS, Swails WS, Driscoll DF et al. Early enteral feeding in postsurgical cancer patients. Fish oil structured lipid-based polymeric formula versus a standard polymeric formula. Ann Surg 1996;223:316–333.

93. Swails WS, Kenler AS, Driscoll DF et al. Effect of a fish oil structured lipid-based diet on prostaglandin release from mononuclear cells in cancer patients after surgery. JPEN J Parenter Enteral Nutr 1997;21:266–274.

94. Sultan J, Griffin SM, Di FF et al. Randomized clinical trial of omega-3 fatty acid-supplemented enteral nutrition versus standard enteral nutrition in patients undergoing oesophagogastric cancer surgery. Br J Surg 2012;99:346–355.

95. Ryan AM, Reynolds JV, Healy L et al. Enteral nutrition enriched with eicosapentaenoic acid (EPA) pre- serves lean body mass following esophageal cancer surgery: results of a double-blinded randomized controlled trial. Ann Surg 2009;249:355–363.

96. Rees D, Miles EA, Banerjee T et al. Dose-related effects of eicosapentaenoic acid on innate immune function in healthy humans: a comparison of young and older men. Am J Clin Nutr 2006;83:331–342.

97. Pontes-Arruda A, Aragao AM, Albuquerque JD. Effects of enteral feeding with eicosapentaenoic acid, gamma-linolenic acid, and antioxidants in mechanically ventilated patients with severe sepsis and septic shock. Crit Care Med 2006;34:2325–2333.

98. Singer P, Theilla M, Fisher H et al. Benefit of an enteral diet enriched with eicosapentaenoic acid and gamma-linolenic acid in ventilated patients with acute lung injury. Crit Care Med 2006;34:1033–1038.

99. Schaffer S, Takahashi K & Azuma J (2000) Role of osmoregulation in the actions of taurine. Amino Acids 2000;19:527–546.

100. Bhavsar TM, Patel SN, Lau-Cam CA. Protective action of taurine, given as a pretreatment or as a posttreatment, against endotoxin-induced acute lung inflammation in hamsters. J Biomed Sci 2010;17(Suppl. 1):S19.

101. Nakajima Y, Osuka K, Seki Y et al. Taurine reduces inflammatory responses after spinal cord injury. J Neuro- trauma 2010;27:403–410.

102. Kaesler S, Sobiesiak M, Kneilling M et al. Effective T-cell recall responses require the taurine transporter Taut. Eur J Immunol 2012;42:831–841.

103. Paauw JD, Davis AT. Taurine concentrations in serum of critically injured patients and age- and sex-matched healthy control subjects. Am J Clin Nutr 1990;52:657–660.

104. American Society of Anesthesiologists Committee. Practice guidelines for preoperative fasting and the use of pharmacologic agents to reduce the risk of pulmonary aspiration: application to healthy patients undergoing elective procedures: an updated report by the American Society of Anesthesiologists Committee on Standards and Practice Parameters. Anesthesiology 2011;114:495–511.

105. Crenshaw JT. Preoperative fasting: will the evidence ever be put into practice? Am J Nurs 2011;111:38–43.

106. van Hoorn DE, Boelens PG, van Middelaar-Voskuilen MC et al. Preoperative feeding preserves heart function and decreases oxidative injury in rats. Nutrition 2005;21:859–866.

107. Bouritius H, van Hoorn DC, Oosting A et al. Carbohydrate supplementation before operation retains intestinal barrier function and lowers bacterial translocation in a rat model of major abdominal surgery. JPEN J Parenter Enteral Nutr 2008;32:247–253.

108. Furnary AP, Zerr KJ, Grunkemeier GL et al. Continuous intravenous insulin infusion reduces the incidence of deep sternal wound infection in diabetic patients after cardiac surgical procedures. Ann Thorac Surg 1999;67:352–360.

109. Rassias AJ, Marrin CA, Arruda J et al. Insulin infusion improves neutrophil function in diabetic cardiac surgery patients. Anesth Analg 1999;88:1011–1016.

110. Berggren H, Ekroth R, Hjalmarson A et al. Enhanced myocardial protection from preoperative carbohydrate infusion in addition to maintained beta- blockade. J Cardiovasc Surg (Torino) 1985;26:454–456.

111. Ljungqvist O, Soreide E. Preoperative fasting. Br J Surg 2003;90:400–406.

112. Nygren J, Thorell A, Jacobsson H et al. Preoperative gastric emptying. Effects of anxiety and oral carbohydrate administration. Ann Surg 1995;222:728–734.

113. Hausel J, Nygren J, Lagerkranser M et al. A carbohydrate-rich drink reduces preoperative discomfort in elective surgery patients. Anesth Analg 2001;93:1344–1350.

114. Hausel J, Nygren J, Thorell A et al. Randomized clinical trial of the effects of oral preoperative carbohydrates on postoperative nausea and vomiting after laparoscopic cholecystectomy. Br J Surg 2005;92:415–421.

115. Melis GC, van Leeuwen PA, von Blomberg-van der Flier BM et al. A carbohydrate-rich beverage prior to surgery prevents surgery-induced immunodepression: a randomized, controlled, clinical trial. JPEN J Parenter Enteral Nutr 2006;30:21–26.

116. Noblett SE, Watson DS, Huong H et al. Pre-operative oral carbohydrate loading in colorectal surgery: a randomized controlled trial. Colorectal Dis 2006;8:563–569.

117. Wang ZG, Wang Q, Wang WJ et al. Randomized clinical trial to compare the effects of preoperative oral carbohydrate versus placebo on insulin resistance after colorectal surgery. Br J Surg 2010;97:317–327.

118. Svanfeldt M, Thorell A, Hausel J et al. Randomized clinical trial of the effect of preoperative oral carbohydrate treatment on postoperative whole-body protein and glucose kinetics. Br J Surg 2007;94:1342–1350.

119. Yuill KA, Richardson RA, Davidson HI et al. The administration of an oral carbohydrate-containing fluid prior to major elective upper-gastrointestinal surgery pre- serves skeletal muscle mass postoperatively – a randomised clinical trial. Clin Nutr 2005;24:32–37.

A novel method for simultaneous measurement of concentration and enrichment of NO synthesis-specific amino acids in human plasma using stable isotopes and LC/MS ion trap analysis

Oosterink JE
Buijs N
Van Goudoever JB
Schierbeek H

Published in:
Journal of Chromatography B 2014; 958: 10-15

abstract

Stable isotope studies offer the opportunity to study the in-depth metabolic pathway of glutamine, citrulline, and arginine, amino acids involved in NO synthesis. The use of multiple stable isotopes can be used to elucidate the exact transformation of glutamine to citrulline and arginine *de novo* synthesis. This novel method provides a purification step using cation exchange resin in combination with a rapid and easy derivatization procedure for a precise and robust measurement of the concentration and isotopic enrichments of NO synthesis-specific amino acids using a liquid chromatography mass spectrometry (LC/MS) ion trap system with high sensitivity and selectivity. The ethyl chloroformate derivatization procedure is beneficial in terms of robustness, velocity, simplicity, and derivative stability. In addition, the ethyl chloroformate derivatization can be performed at room temperature in an aqueous environment without incubation and the isolation of the derivatives from the reaction mixture also serves as a purification step. The concentration and enrichment of NO synthesis-specific amino acids as well as phenylalanine and tyrosine to determine protein turnover, were measured with good inter-day precision for the concentration (<7.4%) and enrichment (<12.7%) in plasma samples at low and high levels. The low limit of quantification was 0.2 $\mu mol \cdot L^{-1}$ for most of the amino acids and the purification method showed to have good recoveries between 78% and 98%. No ion-suppression was observed by post-column infusion experiments. In order to develop new nutritional strategies, this novel method can be used to support the elucidation of the effect of administration of specific supplements on the glutamine–citrulline–arginine pathway by using stable isotope studies.

Introduction

Arginine plays an important role as a regulating metabolite in many different pathways, as well as in protein synthesis and proteolysis and it also serves as the sole precursor for nitric oxide (NO). Since arginine is identified as the precursor for NO and plays important regulating roles in many physiological mechanisms, the metabolism of arginine has been of major interest in medical research in the last decades. Arginine is a conditionally essential amino acid, which means that endogenous arginine production meets the needs of an adult human body in physiological healthy conditions. However, in pathological conditions, arginine levels may decrease due to inadequate *de novo* arginine synthesis. Multiple studies in animals and humans showed that arginine plasma levels are low in several pathological conditions such as cancer, critical illness, after traumatic surgical injury, and during infections (1,2). Under those circumstances, the shortage of arginine may lead to immunosuppression, impaired recovery, disturbed inflammatory response, and diminished vascular function (1–3). Glutamine administration increases plasma levels of arginine and its precursor citrulline (4–7). Stable isotope studies showed that glutamine is used for citrulline formation in the small intestine and that citrulline is the main precursor for *de novo* arginine synthesis in the kidneys (8–10). In addition, the use of multiple stable isotopes made it possible to elucidate the exact contribution of the glutamine molecule to citrulline and arginine formation (11,12). In order to develop new nutritional strategies, the effect of the administration of specific supplements on the glutamine–citrulline–arginine pathway should be determined with stable isotope studies. To measure isotope enrichments of amino acid tracers in plasma samples, gas chromatography mass spectrometry (GC/MS) (13–15), gas chromatography isotope ratio mass spectrometry (GC/C/IRMS) (16–20), liquid chromatography mass spectrometry (LC/MS) (21), and liquid chromatography isotope ratio mass spectrometry (LC/IRMS) (22–24) have been used.

Although the isotope-ratio mass spectrometry (IRMS) techniques are superior for precise isotopic measurement compared with the MS techniques, the IRMS techniques have the disadvantages of being more expensive, being less sensitive, isotope dedicated and requiring an optimal separation of the compounds of interest and other co-eluting peaks for correct measurement. Furthermore, in case of using LC in combination with IRMS, gradient elution of compounds using organic solvents are prohibited since it contains carbon atoms which are oxidized to carbon dioxide. Therefore, methods involving GC/MS analysis are used most

frequently to measure the stable isotope enrichment of amino acids, since this technique has not the disadvantages of the IRMS. However, the detection of arginine and citrulline is difficult with this method and in the last few years LC/MS based methods have been reported to be capable of measuring arginine and citrulline (21). For example, ion-exchange chromatography (25) and normal phase separation principles (26) are used to measure these amino acids. Nowadays, there is an increasing number of reported applications employing reversed-phase LC. The use of ion-pair reagents is an example in which the reagent interacts with the analyte to increase retention in reversed-phase separations (27). Another commonly used method is the chemical modification of the analyte by derivatization, which is widely used in GC/MS analyses for increasing the volatility and polarity and obtaining more sensitivity and selectivity (28). In addition, derivatization of the analyte decreases the matrix effects (29) and increases the height of the isotopic signals measured due to the increase in mass. Especially in isotopic enrichment studies, these features are beneficial. Common reagents for this procedure areophthalaldehyde, phenylisothiocyanate, AccQ·TagTM, alkyl chloroformate, or butanolic HCl (21,28,30–32). However, some of these procedures are extensive and the derivatives obtained with these methods are unstable and long incubation times at different temperatures have to be applied to ensure the success of the derivatization reaction, which is inconvenient. Instead, alkyl chloroformate derivatization procedures used for amino acid analysis with GC/MS are beneficial in terms of robustness, velocity, and derivate stability (30,33). The alkyl chloroformate derivatizationcan be performed at room temperature in an aqueous environment without incubation and the isolation of the derivatives from there action mixture also serves as a purification step. According to Cimlová (33), derivatives obtained with alkyl chloroformates areamenable to LC/MS analysis. In this study, we developed a novel method combining derivatization of NO synthesis-specific amino acids using ethyl chloroformate and LC/MS ion trap analysis for quantification and measurement of isotopic enrichment of these specific amino acids in plasma samples. Additionally, phenylalanine and tyrosine were measured to determine protein turnover.

Materials & Methods

Reference standards

Amino acid standards and L-glutamine, L-citrulline, L-ornithine, L-tyrosine and L-phenylalanine were purchased from Sigma–Aldrich (Milford, NH, USA). [U-$^{13}C_5$] glutamine, l-[Ureido-^{13}C; 3,5-$^{13}C_2$]citrulline, [$^{15}N_2$]ornithine, l-[U-$^{13}C_6$]arginine, [U-$^{13}C_9$]phenylalanine, [U-$^{13}C_9$-^{15}N]tyrosine used as internal standards were purchased from Cambridge Isotope Laboratories (Woburn, MA, USA). The tracers [2-^{15}N]glutamine, [5-^{13}C,D_4]citrulline, and[$^{15}N_2$]arginine, [U-$^{13}C_5$]ornithine, [D_2] tyrosine, [D_4]tyrosine, [D_5]phenylalanine were also purchased from Cambridge Isotope Laboratories (Woburn, MA, USA).

Chemicals

Acetonitrile, ethylacetate, methanol, and formic acid (all HPLC grade or higher) were purchased from Biosolve (Valkenswaard, The Netherlands), ultrapure water was obtained from a Milli-Q purifier (Millipore, Eschborn, Germany). Tridecafluoroheptanoic acid and sulfosalicylic acid (SSA) were purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands) and pyridine, ethanol, ethyl chloroformate, ammonia, hydrochloric acid, and sodium carbonate were purchased from Merck (Darmstadt, Germany). Cation exchange AG-50W-X8 resin 200–400 mesh resin was purchased from Biorad (Veenendaal, The Netherlands).

Sample collection

Blood was collected in heparinized vacuum tubes (Vacutainer; Becton-Dickinson, Franklin Lakes, NJ, USA) and directly placed on ice. After centrifugation (10 minutes, 1910 x g, 4°C), 50 µL of plasma was added to 20 mg dry SSA for deproteinization within 1 h of sampling. After vortex mixing, samples were stored at -80°C until analysis. SSA was used for deproteinization of the catabolic enzymes in the plasma to measure adequate concentrations of the amino acids.

Standard preparation

Standard solutions of 2.5 mmol·L^{-1} were prepared for each amino acid and its labeled isotopomer in 0.1 M HCl and stored at -20°C, except for tyrosine which

was stored at 4°C due to its insolubility at -20°C. A mixture was prepared for the 6 labeled amino acids in purified water which were used as internal standards. A calibration curve was prepared at adequate levels for arginine, citrulline, ornithine, tyrosine, phenylalanine in the range of 5–200 μmol·L^{-1} and for glutamine from 20 to 700 μmol·L^{-1}. Construction of enrichment curves was depending on the enrichments in the sample, but generally it ranges from 0.2% to 21% label.

Sample preparation

20 μL internal standard solution, 20 μL of 1 M HCl, and 200 μL of cation exchange resin (0.1 mg·mL^{-1}) were added to 50 μL of plasma. After mixing and centrifugation (2 minutes, 1600 x g), the supernatant was removed. The resin was washed two times with 1 mL water and the amino acids were extracted in two steps with 500 μL and 200 μL 6 M ammonia. The eluate was dried in a SpeedVac (Genevac Ltd., UK) and re-dissolved in 80 μL acetonitrile, 20 μL 1 M sodium carbonate, 200 μL water, and 140 μL pyridine-ethanol (4:1). Derivatives of the amino acids were prepared by adding 20 μL ethyl chloroformate and were allowed to react for 5 minutes. Then, 400 μL of ethyl acetate was added and the amino acid derivatives were extracted by mixing (1 minute) and centrifugation (2 minutes, 1600 x g). After two extractions with ethyl acetate, the combined solutions of both the first (400 μL) and the second extraction (400 μL) were evaporated under a gentle stream of nitrogen at room temperature until dry. The residue was re-dissolved in 100 μL water/methanol (8:2) and transferred to an insert in an auto sampler vial. Aliquots of 10 μL sample were injected in the LC/MS system.

Liquid chromatographic conditions

Separation of the samples was performed on an ultra-high-performance liquid chromatographic (UHPLC) Thermo Scientific Accela system. The UHPLC Thermo Scientific Accela system was equipped with a degasser, an autosampler with a cooled sample tray, a column oven, and a quaternary pump. Elution was performed at a stable temperature of 40°C using a Waters Acquity BEH C18 column (100 mm X 2.1 mm i.d., 1.7 μm particle size). The mobile phases consisted of 0.5 mM tridecafluoroheptanoic acid (TDFHA) and 1 mL·L^{-1} formic acid (A) and methanol with 0.5 mM TDFHA and1 mL·L^{-1} formic acid (B). Ultra-pure, LC/MS-quality water was used to eliminate excessive background signals and prevent the formation of sodium or potassium adducts. A step-wise gradient starting at

30% B was employed at a flow of 0.2 mL·min^{-1}. From 1 to 4.5 minutes, the % B was linearly increased to 45% and instantly increased to 55% B. Between 4.5 and 24.5 minutes, the gradient was linearly increased to 57.5% B and finally increased to 100% B in 3 minutes with a final hold for 4 minutes. The total run-to-run time (including equilibration prior to injection of the next sample) was 30 minutes. The injection volume was 10 µL and each sample was analyzed in triplicate.

Mass spectrometer instrument settings

The effluent of the UHPLC Thermo Scientific Accela system was directly interfaced to a Linear Ion Trap Velos Pro mass spectrometer (LTQ Velos ProTM) equipped with a heated electrospray interface (Thermo Fisher Scientific, San Jose, CA, USA) operating in the positive mode. The spray voltage of the ion source was set at 3.0 kV and the S-Lens RF Level was set at 68%. The sheath gas was 60 arbitrary units (abu), the auxiliary gas was 20 abu, and the sweep gas was 0 abu. The capillary temperature was set at 300°C. Instrument calibration was performed externally each month with a calibration solution consisting of caffeine, n-butylamine, MRFA (Met-Arg-Phe-Ala), ultramark 1621, acetic acid in a mixture of acetonitrile/water/methanol (2:1:1, v/v). Ion abundance was monitored in the full scan using the zoom-scan mode for glutamine (m/z 245–260), citrulline (m/z 274–290), ornithine (m/z 303–320), arginine (m/z 273–290), phenylalanine (m/z 264–280), and tyrosine(m/z 352–370).

Principle of tracer methodology

Atoms of the same element that differs in mass are called isotopes which are naturally occurring in molecules. Some of the isotopes are stable and by using mass spectrometric analyses the occurrence of the various isotopes of a molecule can be determined. Likewise, the measurement of the relative abundance of isotopes of a compound can be used as a research tool which plays an important role in biological studies nowadays. Stable isotopes of carbon, nitrogen, oxygen and hydrogen are commonly used to incorporate synthetically in molecules of particular interest to form tracers which are given to test subject in order to elucidate biological pathways in the human body. In assumption that enzymes do not discriminate between molecules (tracee) and its labeled counterpart (tracer), the enrichment of the tracer and synthesized products will increase in metabolic processes by which it is involved. Mass spectrometric analysis is used

to determine the enrichment of tracers and enriched synthesized products in biological samples that might be of help to elucidate metabolic pathways. Furthermore, the peak integration of each individual isotope can be used to calculate the enrichment, which is expressed in tracer-to-tracee ratio(TTR) and molar percentage excess (MPE) in this study. The TTR is determined by calculating the ratio of the labeled compound and the unlabeled compound. Additionally, the MPE is the enrichment of that isotope as percentage of all isotopes which is calculated with the next formula:

$$MPE = (TTR / TTR + 1) \times 100\%$$

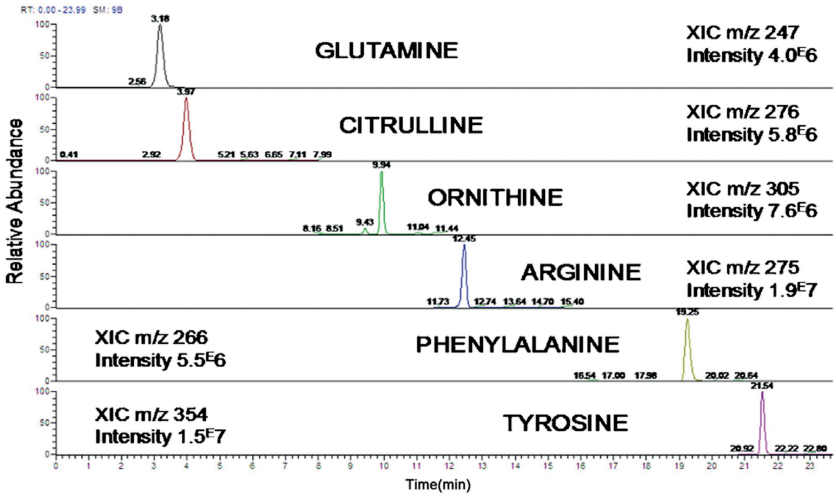

FIGURE 1| Representative extracted ion chromatogram (XIC) of the specific amino acid derivates obtained from a plasma sample which was prepared according the developed purification and derivatization method using ethyl chloroformate as the reagent. The absolute signal intensities are presented in each XIC.

Method validation

The method was validated for the linearity and the limit of quantitation, the intra-day precision, inter-day precision, and recovery and matrix effects. The upper and lower limits of quantitation were determined and defined by the domain of linearity of the concentration and enrichment curves. The intra-day precision was determined by analyzing pool plasma samples in six-fold, which were spiked at relevant enrichment levels. Inter-day precision was evaluated by analyzing

the same pool plasma samples in duplication six different days. Recovery was determined by the analysis of plasma samples that were spiked with known amounts of amino acids before and after sample purification. Matrix effects were evaluated by post-column infusion of internal standards and the injection of and internal standard free matrix to reveal ion suppression.

Results & Discussion

Sample preparation

In preliminary experiments, plasma samples were prepared based on a simple method using a protein precipitation step with acetonitrile and after derivatization the sample extract was analyzed. For this study however, plasma samples were treated with SSA directly after sample collection immediately after sample collection to precipitate proteins and to prevent undesirable side reactions. Although SSA is widely used for protein precipitation, it is not recommended for use in combination with ethyl chloroformate since this derivatization reagent reacts with acids. As a consequence, reaction products were formed by derivatization of SSA in the preliminary experiments, which interfered with the LC/MS analysis. To overcome this problem, a cation exchange purification (14) step was introduced for removing interfering reaction products. Since the amino acids are positively charged and SSA negatively charged at low pH, SSA will not bind to the cation exchange resin and it is removed during the washing steps with water. The introduction of this purification step resulted in lower background signals, which was beneficial for the precise measurement of isotopic enrichments.

Derivatization of amino acids

Since the derivatization method of the amino acid with ethylchloroformate (ECF) is based on preparation methods for GC/MS analyses, which have proven to be robust derivatization methods, the initial conditions of the derivatization were used with some minor modifications (30,34,35). Although the volumes of solvents and reagent were slightly adapted, the ratios were generally maintained. Based on these methods, the amino acids are typically derivatized directly in an alkaline (pH 8) aqueous solution, which is a very fast reaction (5 minutes) catalyzed by pyridine. Derivatization is followed by the extraction of the much less polar formed products using organic solvents. Since the optimal extraction

solvent for arginine and citrulline derivatives was not yet known, the extraction efficiency of the derivatives from there action mixture was investigated to increase the yield. Different extraction solvents were tested such as ethyl acetate, mixtures of hexane/dichloromethane (1:1), and chloroform/iso-octane (2:1). The extraction efficiency for glutamine and citrulline was doubled using ethyl acetate and chloroform/iso-octane (2:1) compared with hexane/dichloromethane (1:1). In the case of arginine, the extraction efficiency was increased more than 10-fold using ethyl acetate.

In conclusion, ethyl acetate had the highest yields and was selected for the extraction of the derivatives in this study.

Liquid chromatography optimization

The UHPLC gradient and mobile phases applied by Cimlová were used in this study as a starting point with small modifications. However, in preliminary experiments that analyzed amino acid ECF derivatives in aqueous solvents, peak tailing was observed after a few injections. Consequently, the back pressure of the column was slowly increased until the upper allowable pressure limit was finally reached, which resulted in an obstructed analytical column. Although replacement of the guard column did not decrease the back pressure of the column, it was suggested that particles such as pyridine salts in the derivatization reaction could be formed and end up in the final extract, causing the increased back pressure. Therefore, the standards were transferred to a 0.2 µm filter to remove possible particles, but peak tailing and increasing backpressure were still observed. With this in mind, it seems that a chemical modification of the column material occurs that involves the derivatization reagent and active groups of the column material, which can be prevented by the addition of ion pair reagents shielding the active groups. By changing the mobile phase composition to formic acid and by the addition of ion pair reagent TDFHA, the peak tailing issues and clogging of the column were solved and numerous injections of standards and samples for amino acid analysis could be performed. Since it is essential that the amino acids are baseline separated from interfering signals to measure the isotopic enrichment accurately, the most suitable gradient was selected based on the maximum distribution of the derivatives over the chromatographic space within 30 minutes for obtaining Gaussian peak shapes for individual compounds (Figure 1).

Mass spectrometry

Optimal ion source parameters for each individual amino acid were obtained by infusion of the amino acid derivatives that were mixed with the HPLC solvent using a PEEK T-piece to mimic the chromatographic conditions. The selectivity of the ion trap VelosPro MS system for reliable isotopic enrichment analysis depends on the resolving power of the instrument. Since high resolving power can discriminate between isotopes, the higher the resolution of the instrument, the better the selectivity for tracer analyses. Therefore, the ion trap MS was operating in the zoom scan that was the optimal compromise between resolution and still having enough data cycles and sensitivity. Another way to improve the precision of the tracer analysis would be to run the instrumental analyses in triplicate and average the isotopic enrichment, which should improve the accuracy of the enrichment in theory by a factor of about the square root of 3.

Method validation

The method was validated for the upper and lower limits of quantitation (LOQ), which were determined by the domain of linearity using the upper and lower part of the calibration curve. As a result, the LOQ for the concentration were in the range of 0.5–700 μmol·L^{-1} (glutamine), 0.2–200 μmol·L^{-1} (ornithine), and 0.2–500 μmol·L^{-1} (arginine, citrulline, phenylalanine and tyrosine). Furthermore, upper and lower LOQ for enrichments were determined for [^{15}N]glutamine (0.50–12% label), [^{13}C-D_4]citrulline(0.28–21% label), [U-$^{13}C_5$]ornithine (0.4–10% label), [$^{15}N_2$]arginine(0.5–14% label), [D_5]phenylalanine (0.7–25% label), [D_2] tyrosine(0.33–22% label), and [D_4]tyrosine (0.08–3% label). Instead of [5-^{13}C,D_4] ornithine, the isotopomer [U-$^{13}C_5$]ornithine was used for the method validation due to availability.The LOQ for the concentration and enrichment were determined and defined by the domain of linearity of the concentration and enrichment curves in the aqueous solvent. Although calibration curves in an aqueous solvent may not account for possible ion suppression effects, it was revealed by post-column infusion that there were no matrix effects at the retention times of the derivatives, as demonstrated in Figure 2. Therefore, the calibration curves were prepared in an aqueous solvent of water/acetonitrile/1 M sodium carbonate (10:4:1) which was of the same composition as the plasma samples just before derivatization. Results of the retention times, molecular ions, and the LOQ for the low and high concentrations are presented in Table 1. Furthermore, an example of a concentration and enrichment curve of arginine are given in Figure 3 and 4.

Figure 3 represent the concentration calibration curve of arginine ranging from 5 to 200 µmol·L^{-1} and the enrichment calibration curve of [$^{15}N_2$]arginine in Figure 4, which is the measured tracer-to-tracee ratio plotted against the percentage of added label to a fixed amount of arginine. The linearity, expressed in R^2, was better than 0.993 for all the calibration curves. The precision and accuracy of the method were evaluated by analyzing pool plasma samples enriched with tracers. The results of the intra-assay precision are less than 5% for each amino acid and its isotopes, which are presented in Tables 1 and 2. The inter-assay precision is less than 15% for all amino acids and their isotopomers, except for [U-$^{13}C_5$]ornithine, due to the low signal of the measured isotopic fragment. The sample preparation method had good recoveries for all amino acids (78–98%), which nicely fits in the recommended 70–120% recovery range.

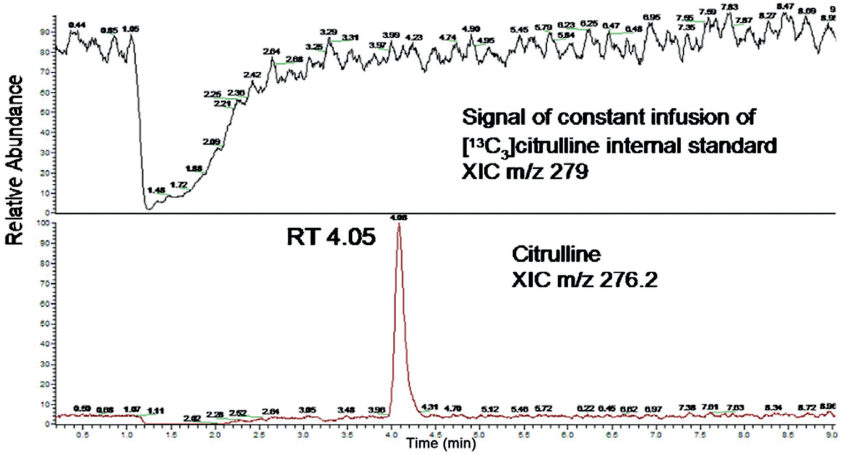

FIGURE 2 | Determination of ion suppression effects by post-column constant infusion of internal standard derivates and simultaneous analysis of a plasma sample prepared according to the developed purification and derivatization method. The extractedion chromatogram (XIC) of citrulline and its internal standard reveals no evident ion suppression or enhancement effects at the retention area of citrulline.

Application of the method in plasma samples

The amino acid kinetics were examined in seven patients. Written informed consent was obtained from all patients. The study protocol was approved by the Medical Ethics Committees of the VU University Medical Center, Amsterdam, The Netherlands and was registered in The Netherlands' trial register (NTR2914). The

patients received 0.5 g·kg^{-1}·day^{-1}. alanyl-glutamine for at least 15 hour prior to the start of the tracer protocol.

TABLE 1 | Validation results amino acid concentration.

Compound	RT (min)	Molecular ion (m/z)	Linear range (µmol·L^{-1})	Linearity (R^2)	Intra-assay precision (%)	Inter-assay precicion (%)	Recovery (%)
Glutamine	3.60	247.2	0.7 - 700	0.9936	2.15	7.39	82
Citrulline	4.05	276.2	0.2 - 500	0.9989	0.46	4.44	92
Ornithine	10.00	305.2	0.2 - 500	0.9989	0.67	4.28	98
Arginine	12.65	275.2	0.2 - 500	0.9983	0.51	5.97	78
Phenylalanine	19.35	266.2	0.2 - 500	0.9993	0.43	4.49	89
Tyrosine	21.63	354.2	0.2 - 500	0.9996	0.61	4.85	88

TABLE 2 | Validation results amino acid enrichment.

Compound	Molecular ion (m/z)	Linear range (% label)	Linearity (R^2)	Enrichment (% MPE)	Inter-assay precicion (%)	Inter-assay precicion (%)
[^{15}N]Glutamine	248.2	0.50–12	0.9994	5.02	1.90	3.22
[^{13}C-D_4]Citrulline	281.2	0.28–21	0.9989	9.35	0.77	5.32
[U-$^{13}C_5$]Ornithine	310.2	0.4–10	0.9977	1.33	3.65	24.61
[$^{15}N_2$]Arginine	277.2	0.5–14	0.9998	4.30	1.41	3.63
[D_5]Phenylalanine	271.2	0.7–25	0.9978	4.91	1.81	12.70
[D_2]Tyrosine	366.2	0.33–22	0.9996	4.17	1.33	5.07
[D_4]Tyrosine	368.2	0.08–3	0.9996	0.70	2.85	9.00

After baseline sampling, a primed, continuous intravenous infusion of the stable isotope tracers was administered and continued for 2.5 h. According to Table 3, tracer dosages for [5-^{13}C,$^2H_4$]citrulline, [$^{15}N_2$]arginine, [2-^{15}N]glutamine, [D_2] tyrosine, [D_4]tyrosine, and [D_5]phenylalanine were simultaneously infused to reach adequate enrichments levels. Furthermore, blood samples were drawn at 0, 30, 60, 90, 120, and 150 minutes after the beginning of the infusion. Significant enrichments in blood samples were found at T = 30, 60, 90, 120, and 150 minutes for [2-^{15}N]glutamine (3–6% MPE)), [5-^{13}C,$^2H_4$]citrulline (3–10% MPE), [^{15}N]citrulline (0.6–6% TTR)), [$^{15}N_2$]arginine (3–11% MPE), and [D_5]phenylalanine (1.8 to 7% MPE). Low enrichments in plasma were found for [^{15}N]arginine (0.4–1.7% TTR) and [5-^{13}C,$^2H_4$]arginine (0.4–1.9% TTR), as well as for [5-^{13}C,$^2H_4$]ornithine (0.5–3% MPE), [D_2]tyrosine (0.7–3.7% MPE), and [D_4]tyrosine (0.2–0.6% MPE). Amino acid

concentrations were found in plasma for glutamine (667 ± 79 μmol·L^{-1}), citrulline (30 ± 4 μmol·L^{-1}), arginine (69 ± 21 μmol·L^{-1}), ornithine (78 ± 24 μmol·L^{-1}), tyrosine (60 ± 17 μmol·L^{-1}), and phenylalanine (63 ± 22 μmol·L^{-1}).

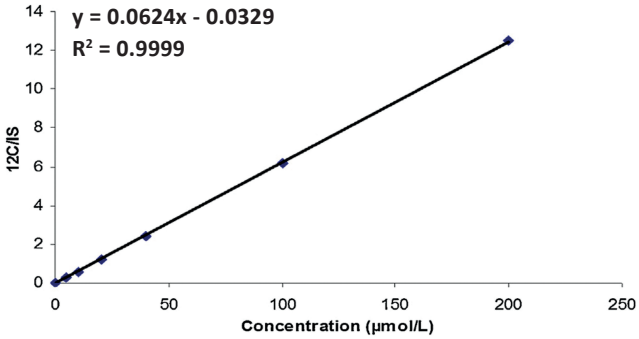

FIGURE 3 | Concentration calibration curve of arginine. The peak area to internal standard ratio is plotted against the concentration of arginine which is ranging from 5 to 200 μmol·L^{-1}. [U-$^{13}C_6$] arginine was used as internal standard. Y = (0.06237 ± 0.00012)*X* - (0.0329 ± 0.0016).

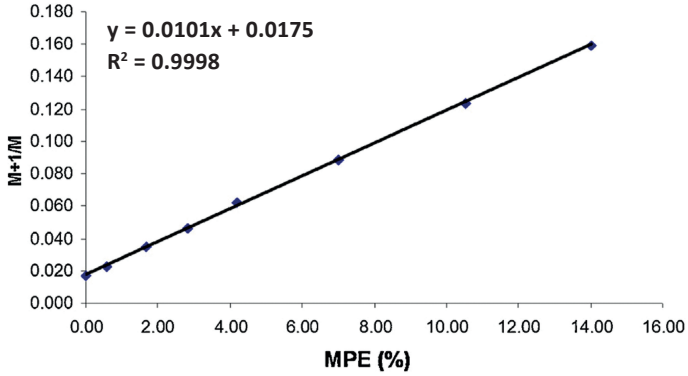

FIGURE 4 | Enrichment calibration curve of [$^{15}N_2$]arginine. The measured tracer-to-tracee ratio is plotted against the percentage of added label which is ranging from 0.5% to 14% label. Y = (0.01011 ± 0.00006)*X* - (0.0175 ± 0.0002).

Conclusion

In this report, a derivatization procedure for amino acids in plasma using ethyl chloroformate in combination with LC/MS ion trap analysis has been discussed. In contrary to GC/MS analysis, this novel method is capable of measuring arginine and citrulline with sufficient sensitivity for enrichment analysis. Despite superior isotopic enrichment analysis of the IRMS, this novel method has the advantage that it is able to measure arginine and citrulline with multiple isotopes like deuterium, 15-nitrogen and 13-carbon in a single run using less sample material. Furthermore, in case of using LC in combination with IRMS, elution of the specific amino acid would be challenging since the use of organic solvents are prohibited. The concentration and enrichment of NO synthesis-specific amino acids as well as phenylalanine and tyrosine to measure protein turnover, were determined with good inter-day precision in plasma samples at low and high enrichment levels. However, a low abundance of [U-$^{13}C_5$]ornithine in the plasma resulted in a lower intra-day precision for enrichment analysis. The purification method had good recoveries (78–98%) and no ion suppression was observed by post-column infusion experiments. Several separation methods using ion-pair reagents and HILIC, as well as derivatization procedures such as the on-line derivatization method of Van Eijk were tested for this study (21). We found that the sensitivity of these methods was insufficient for accurate measurement of low enrichment levels in the plasma samples. The developed ethylchloroformate derivatization procedure provided the highest sensitivity and was only capable of measuring low enrichments levels with good precision in the plasma samples for this study. Another important advantage is that the proposed derivatization procedure is robust, rapid, simple, and can be performed in an aqueous environment without any incubation step and the obtained derivates are stable. All results considered, this novel method provides a rapid and easy derivatization procedure for precise and robust measurement of isotopic enrichments of NO synthesis-specific amino acids using an LC/MS system with high sensitivity and selectivity.

References

1. Morris SM Jr. Arginases and arginine deficiency syndromes. Curr Opin Clin Nutr Metab Care 15(1):64-70.
2. Vissers YL, Dejong CH, Luiking YC, Fearon KC, von Meyenfeldt MF, Deutz NE. Plasma arginine concentrations are reduced in cancer patients: evidence for arginine deficiency? Am J Clin Nutr 2005;81(5):1142-1146.
3. Wu G, Bazer F, Davis T et al. Arginine metabolism and nutrition in growth, health and disease. Amino Acids 2009;37(1):153-168.
4. Houdijk APJ, van Leeuwen PAM, Teerlink T et al. Glutamine-enriched enteral diet increases renal arginine production. JPEN J Parenter Enteral Nutr 1994;18(5):422426.
5. Peters JHC, Wierdsma NJ, Teerlink T, van Leeuwen PAM, Mulder CJJ, van Bodegraven AA. The citrulline generation test: proposal for a new enterocyte function test. Aliment Pharmacol Ther 2008;27(12):1300-1310.
6. Rutten EP, Engelen MP, Wouters EF, Schols AM, Deutz NE. Metabolic effects of glutamine and glutamate ingestion in healthy subjects and in persons with chronic obstructive pulmonary disease. Am J Clin Nutr 2006;83(1):115-123.
7. Ziegler TR, Benfell K, Smith RJ et al. Safety and metabolic effects of L-glutamine administration in humans. JPEN J Parenter Enteral Nutr 1990;14(4 Suppl):137S-146S.
8. Boelens PG, van Leeuwen PAM, Dejong CH, Deutz NE. Intestinal renal metabolism of L-citrulline and L-arginine following enteral or parenteral infusion of L-alanyl-L-[2,15N]glutamine or L-[2,15N]glutamine in mice. Am J Physiol Gastrointest Liver Physiol 2005;289(4):G679-G685.
9. Ligthart-Melis GC, van de Poll MC, Boelens PG, Dejong CH, Deutz NE, van Leeuwen PAM. Glutamine is an important precursor for *de novo* synthesis of arginine in humans. Am J Clin Nutr 2008;87(5):1282-1289.
10. van de Poll MC, Ligthart-Melis GC, Boelens PG, Deutz NE, van Leeuwen PAM, Dejong CH. Intestinal and hepatic metabolism of glutamine and citrulline in humans. J Physiol 2007;581:819-827.
11. Marini JC, Didelija IC, Castillo L, Lee B. Glutamine: precursor or nitrogen donor for citrulline synthesis? Am J Physiol Endocrinol Metab 2010;299(1):E69-79.
12. Tomlinson C, Rafii M, Ball RO, Pencharz P. Arginine synthesis from enteral glutamine in healthy adults in the fed state. Am J Physiol Endocrinol Metab 2011;301(2):E267-E273.
13. Darmaun D, Manary MJ, Matthews DE. A method for measuring both glutamine and glutamate levels and stable isotopic enrichments. Anal Biochem 1985;147(1):92-102.
14. Faure M, Moënnoz D, Montigon F et al. Development of a rapid and convenient method to purify mucins and determine their *in vivo* synthesis rate in rats. Anal Biochem 2002;307(2):244-251.
15. Schierbeek H, van den Akker CHP, Fay LB, van Goudoever JB. igh-precision mass spectrometric analysis using stable isotopes in studies of children. Mass Spectrom Rev 2013;31(2):312-330.
16. Godin JP, Faure M, Breuille D, Hopfgartner G, Fay LB. Determination of 13C isotopic enrichment of valine and threonine by GC-C-IRMS after formation of the N(O,S)-ethoxycarbonyl ethyl ester derivatives of the amino acids. Anal Bioanal Chem 2007;388(4):909-918.

17. Montigon F, Boza JJ, Fay LB. Determination of 13C- and 15N-enrichment of glutamine by gas chromatography/mass spectrometry and gas chromatography/combustion/isotope ratio mass spectrometry after N(O,S)-ethoxycarbonyl ethyl ester derivatisation. Rapid Commun Mass Spectrom 2001;15(2):116-123.
18. Abaye DA, Morrison DJ, Preston T. Strong anion exchange liquid chromatographic separation of protein amino acids for natural 13C-abundance determination by isotope ratio mass spectrometry. Rapid Commun Mass Spectrom 2011;25(3):429-435.
19. Riedijk MA, Stoll B, Chacko S et al. Methionine transmethylation and transsulfuration in the piglet gastrointestinal tract. Proc Natl Acad Sci 2007;104(9):3408-3413.
20. Schaart MW, Schierbeek H, van der Schoor SRD et al. Threonine utilization is high in the intestine of piglets. J Nutr 2005;135(4):765-770.
21. van Eijk HMH, Wijnands KAP, Bessems BAFM, Olde Damink SW, Dejong CH, Poeze M. High sensitivity measurement of amino acid isotope enrichment using liquid chromatography-mass spectrometry. J Chromatogr B 2013;905:31-36.
22. McCullagh J, Gaye-Siessegger J, Focken U. Determination of underivatized amino acid delta(13)C by liquid chromatography/isotope ratio mass spectrometry for nutritional studies: the effect of dietary non-essential amino acid profile on the isotopic signature of individual amino acids in fish. Rapid Commun Mass Spectrom 2008;22(12):1817-1822.
23. Schierbeek H, te Braake F, J Godin JP, Fay LB, van Goudoever JB. Novel method for measurement of glutathione kinetics in neonates using liquid chromatography coupled to isotope ratio mass spectrometry. Rapid Commun Mass Spectrom 2007;21(17):2805-2812.
24. Smith CI, Fuller BT, Choy K, Richards MP. A three-phase liquid chromatographic method for delta13C analysis of amino acids from biological protein hydrolysates using liquid chromatography-isotope ratio mass spectrometry. Anal Biochem 2009;390(2):165-172.
25. Thiele B, Füllner K, Stein N, Oldiges M, Kuhn A, Hofmann D. Analysis of amino acids without derivatization in barley extracts by LC-MS-MS. Anal Bioanal Chem 2008;391(2):2663-2672.
26. Schlichtherle-Cerny H, Affolter M, Cerny C. Hydrophilic interaction liquid chromatography coupled to electrospray mass spectrometry of small polar compounds in food analysis. Anal Chem 2003;75(10):2349-23-54.
27. Piraud M, Vianey-Saban C, Bourdin C et al. A new reversed-phase liquid chromatographic/tandem mass spectrometric method for analysis of underivatised amino acids: evaluation for the diagnosis and the management of inherited disorders of amino acid metabolism. Rapid Commun Mass Spectrom 2005;19(22):3287-3297.
28. Santa T. Derivatization reagents in liquid chromatography/electrospray ionization tandem mass spectrometry. Biomed Chromatogr 2001;25(1-2):1-10.
29. Kostiainen R, Kauppila TJ. Effect of eluent on the ionization process in liquid chromatography-mass spectrometry. J Chromatogr A 2009;1216(4):685-699.
30. Kaspar H, Dettmer K, Gronwald W, Oefner P. Advances in amino acid analysis. Anal Bioanal Chem 2009;393(2):445-52.
31. Johnson DW. Free amino acid quantification by LC-MS/MS using derivatization generated isotope-labelled standards. J Chromatogr B 2011;879(17-18):1345-52.
32. Harder U, Koletzko B, Peissner W. Quantification of 22 plasma amino acids combining derivatization and ion-pair LC-MS/MS. J Chromatogr B 2001;879(7-8):495-504.

33. Cimlová J, Kružberská P, Švagera Z, Hušek P, Šimek P. In situ derivatization-liquid liquid extraction as a sample preparation strategy for the determination of urinary biomarker prolyl-4-hydroxyproline by liquid chromatography-tandem mass spectrometry. J Mass Spectrom 2012;47(3):294-302.
34. Hušek P. Amino acid derivatization and analysis in five minutes. FEBS Lett 1991;280(2):354-356.
35. Huang ZH, Wang J, Gage DA, Watson T, Sweeley CC, Hušek P. Characterization of N-ethoxycarbonyl ethyl esters of amino acids by mass spectrometry. J Chomatogr 1993;635(2):271-281.

Intravenous glutamine supplementation enhances renal *de novo* arginine synthesis in humans: A stable isotope study

Buijs N
Brinkmann SJH
Oosterink JE
Luttikhold J
Schierbeek H
Wisselink W
Beishuizen A
Van Goudoever JB
Houdijk APJ
Van Leeuwen PAM
Vermeulen MAR

4

Published in:
American Journal of Clinical Nutrition 2014; 958: 10-15.

abstract

Background: Arginine plays a role in many different pathways in multiple cell types. Consequently, shortage of arginine, caused by pathological conditions like cancer or injury, has the potential to disturb many cellular and organ functions. Glutamine is the ultimate source for *de novo* synthesis of arginine in humans via the intestinal-renal axis. Therefore, we hypothesized that parenteral glutamine supplementation may stimulate the interorgan pathway of arginine production.

Objective: The objectives were to quantify arginine production from its precursor glutamine and to establish the contribution of the kidneys to *de novo* synthesis of arginine in patients receiving intravenous supplementation of glutamine dipeptide during major abdominal surgery.

Design: Whole-body and renal metabolism of glutamine, citrulline, and arginine was assessed by stable isotopes techniques in 7 patients receiving a perioperative supplement of intravenous alanyl-glutamine (0.5 $g \cdot kg^{-1} \cdot day^{-1}$).

Results: Plasma glutamine, citrulline and arginine concentrations increased significantly in patients receiving intravenous glutamine dipeptide. On whole-body level, 91% of total citrulline turnover was derived from glutamine, whereas 49% of whole-body citrulline turnover was used for *de novo* synthesis of arginine. The kidneys were responsible for 75% of whole-body arginine production from citrulline.

Conclusion: Glutamine and citrulline are important sources for *de novo* arginine synthesis. The kidneys are the main production site for endogenous arginine. After comparison of these results with previous similar studies, our data suggest that an intravenous glutamine supplement doubles renal arginine production from citrulline.

Introduction

Arginine is a conditionally essential amino acid. This means the normal endogenous arginine production meets the need of the adult human body, but in pathophysiological conditions, *de novo* arginine synthesis becomes inadequate. Without adequate supplementation of arginine or its precursors, arginine availability may decrease to concentrations jeopardizing normal biological responses (1,2). Arginine is a molecule of particular interest because it plays a role in many different pathways in multiple cell types. Arginine is an important regulator of protein synthesis and proteolysis and it serves as the precursor for nitric oxide, creatine, agmatine, polyamines, proline and glutamate (3).

Studies in animals and humans showed that arginine becomes an essential amino acid in several pathological conditions, such as after traumatic surgical injury, during cancer, critical illness and infections (4,5). This arginine deficiency may lead to immunosuppression, impaired recovery, disturbed inflammatory response, and diminished vascular function (2, 4). Unfortunately, arginine supplementation showed controversial effects in critically ill patients (6). Yet, it was also found that glutamine administration increases plasma concentrations of arginine (7-10). Metabolic studies showed that glutamine is an important precursor for the synthesis of citrulline in the intestines and arginine by the kidneys (glutamine-citrulline-arginine intestinal-renal axis) (11-13), and also has been quantified using a stable isotope method (12-16). Moreover, glutamine supplementation has positive effects on clinical outcome in several diseases (17-20). It has been suggested that a major part of glutamine's effects can be attributed to the formation of arginine from the administered glutamine (21).

However, data on clearly defined net contribution of glutamine to the synthesis of citrulline and arginine when extra glutamine is administered are lacking. When translating research, the development of optimal nutritional strategies relies on distinct insights into the effect of a supplemental component on metabolic pathways. Especially now that a recently published randomized trial has shown controversial effects of high-dose glutamine supplementation (22), in-depth metabolic data on the effects of glutamine supplementation are desired more than ever. We hypothesized that a parenteral supplement of glutamine, provided as a dipeptide, stimulates citrulline formation and enhances *de novo* arginine synthesis in the kidneys in humans in the postabsorptive state. We designed this stable isotope study in glutamine-supplemented patients undergoing abdominal surgery to investigate the effects of extra glutamine on whole-body and renal metabolism of glutamine, citrulline and arginine.

Subjects & Methods

Patients

Whole-body and renal amino acid kinetics were quantified in 7 patients during abdominal surgery. Patients with parenchymal liver disease, renal failure, inborn metabolic disease, type 1 diabetes, recent weight loss, cachexia, or other indications of metabolic disorders were excluded from the study. After a baseline blood sample was drawn along with a blood sample for routine preoperative laboratory tests, a primed continuous intravenous infusion of 0.5 $g \cdot kg^{-1} \cdot day^{-1}$ alanyl-glutamine (Dipeptiven®, Fresenius Kabi) was administered 1 day before surgery. The dosage of 0.5 g alanyl-glutamine $\cdot kg^{-1} \cdot day^{-1}$ has been proven to be save and is recommended by the manufacturer. Oral intake was allowed only until 12 h before surgery, except for water. Written informed consent was obtained from all patients. The study protocol was approved by the Medical Ethics Committees of the VU University Medical Center, Amsterdam, the Netherlands and was registered in the Netherlands trial register (NTR2914).

Study design

The study design consisted of 2.5-hour tracer infusion and blood sampling during open abdominal surgery enabling steady state isotopomer calculations. The metabolic study was conducted during major abdominal surgery to quantify renal citrulline and arginine turnover. The tracer infusion and the blood sampling were performed during the first exploratory phase of the laparotomy, before invasive surgical intervention of abdominal or retroperitoneal anatomical structures. During the study period, an antecubital vein catheter was used for tracer infusion. This catheter was already in place for alanyl-glutamine infusion and clinical purposes. Blood was sampled from a radial artery catheter, installed according to standard perioperative protocol. In all patients, anesthesia and epidural analgesia was applied according to a standard protocol.

Stable isotope tracers

The tracers [2-^{15}N]glutamine, [5-^{13}C,$^{2}H_4$]citrulline, and [$^{15}N_2$]arginine were purchased from Cambridge Isotope Laboratories. The Department of Clinical Pharmacy at the Erasmus Medical Center in Rotterdam, the Netherlands prepared sterile and pyrogen-free stock solutions of the tracers. The glutamine tracer was

prepared the day before surgery, due to the limited stability of glutamine in solution (72 hour). The stock solutions were diluted with a physiological saline solution just before the start of the tracer infusion.

Tracer infusion, blood sampling, and renal blood flow measurement

After baseline sampling, a primed, continuous intravenous infusion of the stable isotope tracers was administered and continued for 2.5 hour. Tracer infusion was controlled by a calibrated, volume-controlled pump (Graseby 3000; Graseby Medical Ltd).

Blood samples were drawn at 30, 60, 90, 120, and 150 minutes after the beginning of the tracer infusion. After approximately 120 minutes, at isotopic steady state, blood was drawn from both the radial artery catheter and the renal vein by direct puncture simultaneously, to study renal metabolism. The renal vein was sampled before organ clamping or transection.

Blood was collected in heparinized vacuum tubes (Vacutainer; Becton-Dickinson) and placed on ice. First, the hematocrit of the blood samples was measured. Blood was centrifuged (10 minutes, 1910 x g, 4°C), and 50 µL of the supernatant was deproteinized with 20 mg dry SSA within 1 hour after sampling. After mixing, samples were stored at -80°C until analysis.

To quantify renal amino acid fluxes, renal blood flow was determined with a color Doppler ultrasound (Aloka Prosound SSD 5000; Aloka Co, Ltd) as described previously (12); time-averaged mean velocity of the bloodstream and cross-sectional area of the right renal vein were measured during the explorative phase of the operation. Blood flow was calculated by multiplying the cross-sectional area with the velocity of the bloodstream. Plasma flow was calculated by this equation: plasma flow = blood flow(1-hematocrit). Total renal flow was estimated by multiplying plasma flow by 2. Mean renal plasma flow was used to calculate amino acid fluxes across the kidneys.

Mass spectrometric analysis

Plasma enrichments of the infused tracers and the tracer products were measured by liquid chromatography-mass spectrometry (LC/MS) (23). Briefly, 20 µL of hydrochloric acid, 20 µL of intern standard and 200 µL cation exchange solution [0.1 mg·mL^{-1} AG 50W-X8 resin (Biorad), 200-400 mesh] were added to

50 µL of deproteinized plasma. After mixing and centrifugation, the supernatant was removed. The resin was washed with 1 mL water and the amino acids were extracted twice with 500 µL and 200 µL 6 mol·L^{-1} ammonia respectively. The eluate was dried in a SpeedVac (Genevac Ltd) and redissolved in 80 µL acetonitrile, 20 µL 1 mol·L^{-1} sodium carbonate, 200 µL water and 140 µL pyridine-ethanol (4:1). Derivatives of the amino acids were prepared by adding ethyl chloroformate and incubating for 5 minutes. After two extractions with ethyl acetate, the combined solutions of both the first (400 µL) and the second extraction (400 µL) were evaporated under a gentle stream of nitrogen at room temperature until it was almost dry and redissolved in 100 µL 20% methanol. Analyses were performed on a LC/MS (Velos Pro, Thermo Fisher) by injecting 10 µL of sample extract on a 2.1- to100 mm, 1.7µm Waters Acquity BEH C18 column. Elution was performed at a stable temperature of 40˚C using mobile phases consisting of 0.5 mmol·L^{-1} tridecafluoroheptanoic acid and 1 mL·L^{-1} formic acid (phase A) and methanol with 0.5 mmol·L^{-1} tridecafluoroheptanoic acid and 1 mL·L^{-1} formic acid (phase B). Ion abundance was monitored in full scan using the zoom-scan modus for glutamine (m/z 245-260), citrulline (m/z 274-290), ornithine (m/z 303 - 320) and arginine (m/z 273 -290).

Calculations

All equations used for calculating the whole-body and organ metabolism of glutamine, citrulline and arginine are described in Table 1. Isotope enrichments were expressed as mole percent excess (MPE), calculated as enrichment at steady state minus isotopic background measurements at baseline. Whole-body turnover of glutamine, citrulline and arginine was calculated. Furthermore, whole-body conversion rates for glutamine into citrulline and citrulline into arginine were determined. Renal plasma flow and arteriovenous differences in amino acid concentrations and isotopic enrichments were used to obtain insight into the renal metabolism of citrulline and arginine. Arginine production from citrulline in the kidneys was calculated by using the arterial enrichment of citrulline [M+5] and the venous enrichment of arginine [M+5]. The renal output of arginine [M+5] was corrected for bypassing arginine. All fluxes are presented in micromole amino acid per kilogram of total body weight of the human subject per hour.

TABLE 1 | Equations.

Variable	Equation
Whole-body Turnover	$Q = i[(E_i/E_p) - 1]$
Whole-body endogenous glutamine flux	$Q_{Gln\text{-}endo} = \text{Total } Q_{Gln} - Q_{Gln\text{-}exo}$
Whole-body conversion rate glutamine to citrulline	$Q_{Gln\text{-}Cit} = E_{Cit\ M+1}/E_{Gln\ M+1} \times Q_{Cit\ M+5}$
Whole-body conversion rate citrulline to arginine	$Q_{Cit\text{-}Arg} = E_{Arg\ M+5}/E_{Cit\ M+5} \times Q_{Arg\ M+2}$
Whole-body conversion rate glutamine to arginine	$Q_{Gln\text{-}Arg} = E_{Arg\ M+1}/E_{Gln\ M+1} \times Q_{Arg\ M+2}$
Renal Net Balance (NB)	$NB = ([A]\text{-}[V]) \times F$
Renal Tracer Net Balance (TNB)	$TNB = ((E_A\ [A])\text{-}(E_V[V]) \times F$
Renal Fractional Extraction (FE)	$FE = \text{Tracer NB} / ([A] \times E_A \times F)$
Renal Disposal	TNB/E_V
Renal Production	Net renal influx - NB
Renal Arginine_{M+5} Output	$([V] \times E_V \times F) - ([A] \times E_A \times F \times (1\text{-}FE))$
Renal arginine production from citrulline	$Q_{Citrulline \rightarrow Arginine} = \text{Arginine}_{M+5}\text{Output} / ([A_{Cit}] \times E_{A\text{-}Cit\ M+5} \times F) \times ([A_{Cit}] \times F)$

Q, flux in μmol·kg^{-1}·h^{-1}. E, enrichment in mole percent excess (MPE). E_i, enrichment in infusate. E_p, enrichments in plasma at steady state. $Q_{Gln\text{-}endo}$, endogenous glutamine flux in μmol·kg^{-1}·h^{-1}. Total Q_{Gln}, whole-body glutamine turnover during alanyl-glutamine infusion in μmol·kg^{-1}·h^{-}. $Q_{Gln\text{-}exo,}$ exogenous flux of glutamine in μmol·kg^{-1}·h^{-}. $Q_{Gln\text{-}Cit}$'', whole-body glutamine to citrulline conversion rate in μmol·kg^{-1}·h^{-1}. $E_{Cit\ M+1}$, plasma enrichment of citrulline [M+1] in MPE. $E_{Gln\ M+1}$, plasma enrichment of glutamine [M+1] in MPE. $Q_{Cit\ M+5}$, whole-body flux of citrulline [M+5] μmol·kg^{-1}·h^{-1}. $Q_{Cit\text{-}Arg}$, whole-body citrulline to arginine conversion rate in μmol·kg^{-1}·h^{-1}. $E_{Arg\ M+5}$, plasma enrichment of arginine [M+5] in MPE. $E_{Cit\ M+5,}$ plasma enrichment of citrulline [M+5] in MPE. $Q_{Arg\ M+2}$, whole-body flux of arginine [M+2] in μmol·kg^{-1}·h-^{1}. NB, renal net balance in μmol·kg^{-1}·h^{-1}. TNB, renal tracer net balance in μmol·kg^{-1}·h^{-1}. FE, renal fractional extraction in percent. F, renal plasma flow. [A], arterial concentration. [V], venous concentration. E_A, arterial enrichment in MPE. E_V, venous enrichment.

Statistical analysis

Results are presented in mean ± SEM. Enrichment curves were fitted to determine a mean steady state value per individual using Prism 5.0 for Windows (GraphPad Software Inc). Quantile-quantile plots of the data showed linearity, indicating that the distribution is consistent with the assumption of normality. The Student's *t*-test was used to determine significant differences in amino acid concentrations between the plasma sample before the start of parenteral alanyl-glutamine administration and during parenteral alanyl-glutamine supplementation just before the tracer infusion. The 1-sample *t* test was used to test whether the values were significantly

different from zero. SPSS 20.0 for Windows software (SPSS Inc) was used to perform statistical tests. $P<0.05$ was considered to indicate a significant difference.

Results

Baseline characteristics of the patients are shown in Table 2. The patients received 0.5 g alanyl-glutamine $\cdot kg^{-1} \cdot day^{-1}$ for at least 15 h before the start of the tracer protocol. Tracer dosage for [5-^{13}C,$^{2}H_4$]citrulline and [$^{15}N_2$]arginine were comparable with that in other studies (11, 18, 21); [2-^{15}N]glutamine tracer dosage was corrected for the simultaneous alanyl-glutamine infusion to reach adequate enrichments (Table 3). We found significant enrichments of glutamine [M+1], citrulline [M+5] and citrulline [M+1] and arginine [M+2]. We also found significant enrichments of arginine in plasma at [M+1] and [M+5], which confirms the metabolic route from glutamine to citrulline and arginine. No significant tracer enrichments for ornithine were detected. Arterial plasma enrichments of the infused tracers and the tracer products were observed to be in steady state (Figure 1).

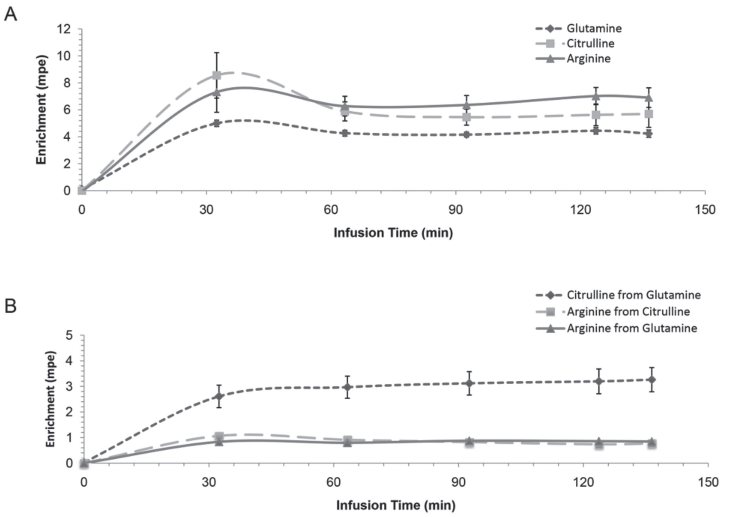

FIGURE 1 | Steady state curve of primed, continuously infused tracers (A) and their products (B) in MPE (n=7). 60 minutes after the start of tracer infusion, all isotopomers are in equilibrium.

Amino acid concentrations increase with a supplement of glutamine

The plasma concentrations of glutamine, citrulline and arginine significantly increased after the administration of intravenous 0.5 g alanyl-glutamine ·kg^{-1}· day^{-1} compared to baseline (Figure 2).

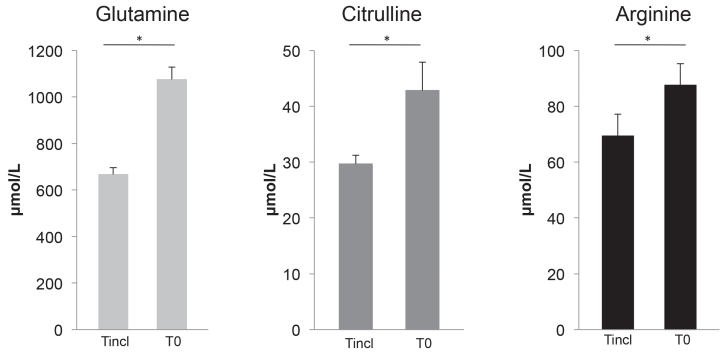

FIGURE 2 | Plasma concentrations of glutamine, citrulline and arginine (mean ± SEM) at the time of inclusion (Tincl) and after the administration of intravenous 0.5 g·kg^{-1}·day^{-1} alanyl-glutamine just before the start of the tracer infusion (T0) (n=7). The Student's t-test was used to determine significant differences in amino acid concentrations between Tincl and T0. *P < 0.05.

Whole-body amino acid turnover

Whole-body plasma turnover of glutamine, citrulline and arginine was 423±29, 9.5±1.0 and 30.4±2.2 µmol·kg^{-1}·h^{-1}, respectively. Whole-body endogenous glutamine flux was 327±29 µmol·kg^{-1}·h^{-1}. Estimated whole-body citrulline production from plasma glutamine was 8.1±0.9 µmol·kg^{-1}·h^{-1}, representing 91% of the total citrulline turnover. Forty-nine percent of total citrulline turnover was used for *de novo* arginine synthesis at the whole-body level, which was 4.5±0.6 µmol·kg^{-1}·h^{-1}. Whole-body arginine [M+1] production derived from glutamine [M+1] was 6.33 µmol·kg^{-1}·h^{-1}. Since this is more than 100% of the citrulline to arginine conversion rate, this is probably an overestimate caused by nitrogen recycling or channeling, as discussed later.

TABLE 2 | Patient baseline characteristics.

Patient characteristics	Values (n=7)
Sex	2 female; 5 male
Age (years)	64 ± 3
Height (cm)	178 ± 3
Weight (kg)	91 ± 5
Body Mass Index	29 ± 1
Amino Acid Concentration ($\mu mol \cdot L^{-1}$)*	
Glutamine	667 ± 30
Citrulline	30 ± 2
Arginine	69 ± 8
Preoperative Laboratory Results	
Creatinine ($\mu mol \cdot L^{-1}$)	84 ± 9
Urea ($mmol \cdot L^{-1}$)	6 ± 0.5
Glomerular Filtration Rate	>60
Billirubin ($\mu mol \cdot L^{-1}$)	7 ± 2
Albumin ($g \cdot L^{-1}$)	41 ± 3
Glucose ($mmol \cdot L^{-1}$)	6.9 ± 0.6
Surgical procedure	7 Infrarenal abdominal aorta aneurysm repair
Urine production during protocol (mL)	156 ± 40
Total fluid provided during protocol (mL)	1489 ± 199
Total renal Plasma flow ($mL \cdot kg^{-1} \cdot min^{-1}$)	5.58 ± 0.5

Values in mean ± SEM. *Before alanyl-glutamine administration

TABLE 3 | Tracer dosage.

Tracer	Priming dose ($\mu mol \cdot kg^{-1}$)	Infusion dose ($\mu mol \cdot kg^{-1} \cdot h^{-1}$)
L-[2-^{15}N]glutamine	23.82	20.98
L-[5-^{13}C-4,4,5,5$^{2}H_4$]citrulline	1.18	0.57
L-[$^{15}N_2$]arginine	2.73	2.16

Renal metabolism

To determine organ-specific amino acid handling, the enrichment of simultaneously taken arterial and venous samples was used. Arterial and venous plasma enrichments and concentrations are summarized in table 4. Renal net balance of citrulline showed an uptake of 4.1±1.2 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ and this was

accompanied by a net release of arginine of 4.0±0.7 μmol·kg^{-1}·h^{-1}, illustrating the role of the kidney in arginine metabolism (Figure 3). Eighty-five percent of the net renal citrulline [M+5] uptake was utilized for net arginine [M+5] production. Total renal arginine production from citrulline was 3.0±0.7 μmol·kg^{-1}·h^{-1}, representing 72% of net renal citrulline uptake and 74% of net renal arginine release. The kidneys were responsible for 75% of *de novo* arginine synthesis at the whole-body level.

Discussion

This study shows the qualitative and quantitative effects of an intravenous supplement of glutamine on whole-body and renal metabolism of glutamine, citrulline and arginine in humans in the postabsorptive state. The use of the [2-^{15}N]glutamine tracer showed that almost all circulating citrulline derived from glutamine. Approximately half of the circulating plasma citrulline was used for arginine production. The kidneys were responsible for 75% of whole-body *de novo* arginine synthesis with a production rate of approximately 3.0 μmol·kg^{-1}·h^{-1}.

This study's effect of the intravenous glutamine supplement on the metabolism of citrulline formation and arginine production should be interpreted in the background of results obtained in previous stable isotope studies with a similar design. The study by Ligthart-Melis et al is comparable to the current study, because similar intravenous stable isotopes were used in human subjects in the postabsorptive state during abdominal surgery to determine renal metabolism (12). In those patients, the whole-body glutamine flux was 240±14 μmol·kg^{-1}·h^{-1}, whereas in our patients receiving a supplement of glutamine, the whole-body total glutamine flux was almost doubled and the endogenous glutamine flux was approximately 40% higher. In both studies, half of circulating citrulline was used for *de novo* arginine synthesis. However, in our study the glutamine supplement caused an increase in citrulline concentrations and renal arginine production from citrulline was 3.0±0.7 μmol·kg^{-1}·h^{-1} compared with only 1.5±0.7 μmol·kg^{-1}·h^{-1} in the study without glutamine administration (12). Thus, renal arginine production seems to be doubled in humans receiving 0.5 g alanyl-glutamine·kg^{-1}·day^{-1} intravenously, compared to previous results in humans without glutamine supplementation. This was also reflected by the significant increase of arginine plasma concentration during glutamine infusion compared with baseline in the current study.

TABLE 4 | Renal arterial and venous plasma amino acid concentrations and tracer enrichments.

	Citrulline ($\mu mol \cdot L^{-1}$)	Arginine ($\mu mol \cdot L^{-1}$)	Citrulline [M+5] (MPE)	Arginine [M+2] (MPE)	Arginine [M+5] (MPE)
Arterial	33 ± 7	80 ± 7	6.11 ± 1.0	6.9 ± 0.8	0.7 ± 0.1
Venous	21 ± 4	91 ± 9	6.3 ± 1.0	5.9 ± 0.7	1.1 ± 0.1

Values in mean ± SEM (n=7). Amino acid concentrations and enrichments were measured by using mass spectrometry.

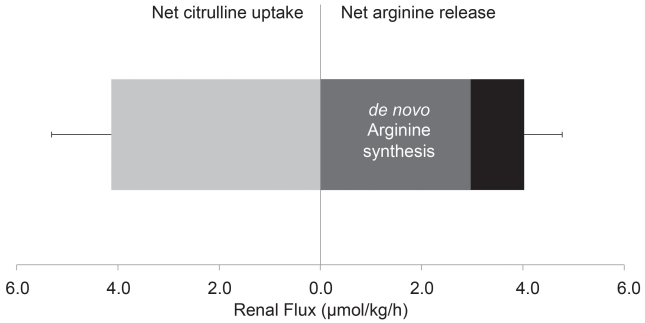

FIGURE 3 | Renal net citrulline uptake and net arginine release in $\mu mol \cdot kg^{-1} \cdot h^{-1}$ (n=7), from which 74% was derived from *de novo* arginine synthesis from citrulline (dark grey).

Arginine is an important regulator of the immune system, cell homeostasis and protein synthesis and it plays important roles in multiple metabolic pathways (24). In addition, arginine is the sole precursor for nitric oxide generation, a signaling agent with a crucial role in immunity, inflammation and organ perfusion (25). As demonstrated in previous studies and the present study, citrulline can be converted into arginine, mainly in the kidneys (12,26). This *de novo* arginine synthesis has been shown to be the main regulator of plasma arginine concentrations (11). Plasma arginine concentrations can be affected in pathological conditions in which the disposal capacity of arginine is enhanced (5,27-31). Thus, in various pathological conditions *de novo* arginine synthesis appears to be insufficient. Systemic arginine deficiency contributes to immunosuppression, inflammation disorders and vascular dysfunction in sick patients, which may lead to concomitant morbidity and mortality (4). Metabolic studies suggested that an arginine deficiency is related to a decreased glutamine availability, affecting intestinal citrulline formation and subsequently arginine production (11). Conversely, after glutamine supplementation, an increase in citrulline and arginine plasma

concentrations is found (7,10,32). Other intermediates of the intestinal-renal axis have been suggested to enhance arginine production as well. For example, the supplementation of ornithine α-ketoglutarate resulted in increased citrulline and arginine concentrations, whereas ornithine supplementation did not (33). Furthermore, citrulline regulates *de novo* arginine synthesis and citrulline supplementation could augment a similar effect on arginine production, as shown in the current study. However, glutamine is still considered to be the premier precursor of arginine via the intestinal-renal axis. In fact, in this study we confirmed the existence of this precursor role and demonstrated that glutamine supplementation enhances renal *de novo* arginine synthesis from citrulline in comparison to previous published results (12).

Glutamine administration showed to be beneficial in several pathological conditions (7,10). Recently the administration of high doses of glutamine in critically ill patients with multi-organ failure became controversial after possible adverse effects were found in these patients (22).

However, this clinical trial included patients with kidney failure and liver failure, which are both contraindications for glutamine supplementation. After combination of available studies on glutamine supplementation, however, it was found that glutamine still may prevent and limit infections, improve recovery from injury and positively affect mortality (34-36). Because glutamine is the substrate for citrulline and arginine, (part of) the effects of glutamine supplementation could be mediated by its derivatives. As described before, *de novo* arginine production from citrulline is the regulating factor in optimizing plasma arginine concentrations in the body. Excessive arginine supplementation could also have adverse effects, probably from the excessive formation of nitric oxide and oxidative metabolites, subsequently leading to oxidative stress (6). Glutamine supplementation is suggested to be a more physiological way of correcting arginine concentrations and subsequently achieve both glutamine and arginine benefits. In light of previous work, our results seem to support this by showing the qualitative and quantitative effects of the glutamine supplement on renal *de novo* arginine synthesis from citrulline.

Some specific patient populations may benefit most from a supplement of glutamine, because they show disease-related low glutamine and arginine concentrations. In combination with data from the literature, our results indicate that in these specific patient populations, intravenous glutamine supplementation could restore renal arginine production.

However, the aim of this study was to investigate the effect of glutamine administration on arginine production on whole-body level as well as in the kidneys under the most physiological condition possible. The included patients did not have metabolic disorders, organ failures or aberrant diets, glutamine, citrulline and arginine concentrations were in the normal ranges, and the tracer protocol was conducted in the exploratory phase of surgery. Although our results suggest that an intravenous supplement of glutamine could correct depleted arginine concentrations in patients with a pathological induced arginine deficiency, quantitative and qualitative studies using a stable isotope technique in these specific patient populations should be performed in the future. Furthermore, the patients in this study had an average high BMI and were relatively of high age, representative of patients with abdominal aortic aneurysm, which may have influenced our metabolic measurements.

The use of stable isotope tracers to quantify glutamine to arginine metabolism has been a subject of discussion in the past years, ever since a study by Marini et al. showed that the use of a [2-^{15}N]glutamine tracer may overestimate the quantitative contribution of glutamine to arginine in mice due to nitrogen recycling (15). However, studies in humans showed that citrulline plasma concentrations increase after glutamine supplementation, which is confirmed by our study. This is possible only when a substantial part of the carbon skeleton of glutamine is used for citrulline formation. An excellent study by Tomlinson et al. evaluated this complexity of the glutamine to arginine pathway in humans in the fed state by using both a C-labeled and an N-labeled glutamine tracer (16). Similar to our results, they found that the N-labeled tracer showed a contribution of glutamine [M+1] to arginine [M+1] synthesis that exceeded the citrulline to arginine conversion. Furthermore, they demonstrated that the labeled N-atom was found in various locations of the ornithine, citrulline and arginine molecules. Thus, although they demonstrated that the N-atom from glutamine is used for ornithine formation and the equivalent isotopomers of citrulline and arginine, the way it contributes to the formation of the isotopomers remains indefinite. Our results showed citrulline [M+1] and arginine [M+1] enrichments, but ornithine enrichments were not significant, indicating a complex contribution of the N-atom in this pathway. Consequently, we agree with Tomlinson et al. (16) that the N-labeled glutamine tracer can provide qualitative information on the glutamine to citrulline and arginine pathway, yet quantitative results should be interpreted with caution because outcomes may overestimate the contribution of glutamine to citrulline and arginine formation. More important, quantitatively they

confirmed with a [1-^{13}C]glutamine tracer that the carbon skeleton of glutamine is used for approximately 50% of *de novo* arginine synthesis in humans, supporting the existence of the glutamine-citrulline-arginine pathway (16). With this in mind, future stable isotope studies to elucidate glutamine to arginine metabolism in humans should be performed using a carbon-labeled glutamine tracer.

Conclusion

This study was conducted to investigate the way in which a therapeutic dose of parenteral glutamine affects the synthesis of arginine at the whole-body level and in the kidneys specifically. Although the amino acid kinetics were examined in only seven patients, our data consistently showed that during glutamine supplementation most of the circulating citrulline derived from glutamine and 49% is used for *de novo* arginine synthesis in the kidneys. The intravenous glutamine supplement resulted in significantly higher glutamine, citrulline and arginine plasma concentrations. To our knowledge, this is the first quantitative study showing that renal arginine production from citrulline is enhanced in patients receiving an intravenous glutamine supplement when comparing these results with previously published data. In conclusion, an intravenous supplement of glutamine dipeptide enhances *de novo* arginine synthesis in the kidneys of humans in the postabsorptive state.

References

1. Nieves C, Jr., Langkamp-Henken B. Arginine and immunity: a unique perspective. Biomed Pharmacother 2002;56:471-82.
2. Wu G, Bazer FW, Davis TA et al. Arginine metabolism and nutrition in growth, health and disease. Amino Acids 2009;37:153-68.
3. Morris SM, Jr. Arginine metabolism: boundaries of our knowledge. J Nutr 2007;137:1602S-9S.
4. Morris SM, Jr. Arginases and arginine deficiency syndromes. Curr Opin Clin Nutr Metab Care 2012;15:64-70.
5. Vissers YL, Dejong CH, Luiking YC, Fearon KC, von Meyenfeldt MF, Deutz NE. Plasma arginine concentrations are reduced in cancer patients: evidence for arginine deficiency? Am J Clin Nutr 2005;81:1142-6.
6. Marik PE, Zaloga GP. Immunonutrition in critically ill patients: a systematic review and analysis of the literature. Intensive Care Med 2008;34:1980-90.
7. Houdijk AP, Rijnsburger ER, Jansen J, Wesdorp RI, Weiss JK, McCamish MA, Teerlink T, Meuwissen SG, Haarman HJ, Thijs LG et al. Randomised trial of glutamine-enriched enteral nutrition on infectious morbidity in patients with multiple trauma. Lancet 1998;352:772-6.
8. Peters JH, Wierdsma NJ, Teerlink T, van Leeuwen PA, Mulder CJ, van Bodegraven AA. The citrulline generation test: proposal for a new enterocyte function test. Aliment Pharmacol Ther 2008;27:1300-10.
9. Rutten EP, Engelen MP, Wouters EF, Schols AM, Deutz NE. Metabolic effects of glutamine and glutamate ingestion in healthy subjects and in persons with chronic obstructive pulmonary disease. Am J Clin Nutr 2006;83:115-23.
10. Ziegler TR, Benfell K, Smith RJ, Young LS, Brown E, Ferrari-Baliviera E, Lowe DK, Wilmore DW. Safety and metabolic effects of L-glutamine administration in humans. JPEN J Parenter Enteral Nutr 1990;14:137S-46S.
11. Deutz NE. The 2007 ESPEN Sir David Cuthbertson Lecture: amino acids between and within organs. The glutamate-glutamine-citrulline-arginine pathway. Clin Nutr 2008;27:321-7.
12. Ligthart-Melis GC, van de Poll MC, Boelens PG, Dejong CH, Deutz NE, van Leeuwen PA. Glutamine is an important precursor for *de novo* synthesis of arginine in humans. Am J Clin Nutr 2008;87:1282-9.
13. van de Poll MC, Ligthart-Melis GC, Boelens PG, Deutz NE, van Leeuwen PA, Dejong CH. Intestinal and hepatic metabolism of glutamine and citrulline in humans. J Physiol 2007;581:819-27.
14. Ligthart-Melis GC, van de Poll MC, Dejong CH, Boelens PG, Deutz NE, van Leeuwen PA. The route of administration (enteral or parenteral) affects the conversion of isotopically labeled L-[2-15N]glutamine into citrulline and arginine in humans. JPEN J Parenter Enteral Nutr 2007;31:343-8.
15. Marini JC, Didelija IC, Castillo L, Lee B. Glutamine: precursor or nitrogen donor for citrulline synthesis? Am J Physiol Endocrinol Metab 2010;299:E69-E79.
16. Tomlinson C, Rafii M, Ball RO, Pencharz P. Arginine synthesis from enteral glutamine in healthy adults in the fed state. Am J Physiol Endocrinol Metab 2011;301:E267-E273.
17. Avenell A. Hot topics in parenteral nutrition. Current evidence and ongoing trials on the use of glutamine in critically-ill patients and patients undergoing surgery. Proc Nutr Soc 2009;68:261-8.

18. Kuhn KS, Muscaritoli M, Wischmeyer P, Stehle P. Glutamine as indispensable nutrient in oncology: experimental and clinical evidence. Eur J Nutr 2010;49:197-210.
19. Marik PE, Flemmer M. Immunonutrition in the surgical patient. Minerva Anestesiol 2012;78:336-42.
20. Noe JE. L-glutamine use in the treatment and prevention of mucositis and cachexia: a naturopathic perspective. Integr Cancer Ther 2009;8:409-15.
21. Vermeulen MA, van de Poll MC, Ligthart-Melis GC, Dejong CH, van den Tol MP, Boelens PG, van Leeuwen PA. Specific amino acids in the critically ill patient--exogenous glutamine/arginine: a common denominator? Crit Care Med 2007;35:S568-S576.
22. Heyland D, Muscedere J, Wischmeyer PE, Cook D, Jones G, Albert M, Elke G, Berger MM, Day AG. A randomized trial of glutamine and antioxidants in critically ill patients. N Engl J Med 2013;368:1489-97.
23. Oosterink JE, Buijs N, van Goudoever JB, Schierbeek H. A novel method for simultaneous measurement of concentration and enrichment of NO synthesis-specific amino acids in human plasma using stable isotopes and LC/MS ion trap analysis. J Chromatogr B Analyt Technol Biomed Life Sci 2014;958:10-5.
24. Morris SM, Jr. Arginine: beyond protein. Am J Clin Nutr 2006;83:508S-12S.
25. Luiking YC, ten Have GA, Wolfe RR, Deutz NE. Arginine *de novo* and nitric oxide production in disease states. Am J Physiol Endocrinol Metab 2012;303:E1177-E1189.
26. Ligthart-Melis GC, van de Poll MC, Vermeulen MA, Boelens PG, van den Tol MP, van Schaik C, de Brandt JP, Deutz NE, DeJong CH, van Leeuwen PA. Enteral administration of alanyl-[2-(15) N]glutamine contributes more to the *de novo* synthesis of arginine than does intravenous infusion of the dipeptide in humans. Am J Clin Nutr 2009;90:95-105.
27. Baylis C. Arginine, arginine analogs and nitric oxide production in chronic kidney disease. Nat Clin Pract Nephrol 2006;2:209-20.
28. Davis JS, Anstey NM. Is plasma arginine concentration decreased in patients with sepsis? A systematic review and meta-analysis. Crit Care Med 2011;39:380-5.
29. Luiking YC, Poeze M, Ramsay G, Deutz NE. Reduced citrulline production in sepsis is related to diminished *de novo* arginine and nitric oxide production. Am J Clin Nutr 2009;89:142-52.
30. Nijveldt RJ, Prins HA, Siroen MP, Rauwerda JA, Teerlink T, van Leeuwen PA. Low arginine plasma levels in patients after thoracoabdominal aortic surgery. Eur J Clin Nutr 2000;54:615-7.
31. Pribis JP, Zhu X, Vodovotz Y, Ochoa JB. Systemic arginine depletion after a murine model of surgery or trauma. JPEN J Parenter Enteral Nutr 2012;36:53-9.
32. Houdijk AP, van Leeuwen PA, Teerlink T, Flinkerbusch EL, Boermeester MA, Sauerwein HP, Wesdorp RI. Glutamine-enriched enteral diet increases renal arginine production. JPEN J Parenter Enteral Nutr 1994;18:422-6.
33. Cynober L, Coudray-Lucas C, De Bandt JP, Guechot J, Aussel C, Salvucci M, Giboudeau J. Action of ornithine alpha-ketoglutarate, ornithine hydrochloride, and calcium alpha-ketoglutarate on plasma amino acid and hormonal patterns in healthy subjects. J Am Coll Nutr 1990;9:2-12.
34. Bollhalder L, Pfeil AM, Tomonaga Y, Schwenkglenks M. A systematic literature review and meta-analysis of randomized clinical trials of parenteral glutamine supplementation. Clin Nutr 2013;32:213-23.

35. Clinical Evaluation Research Unit. Canadian Clinical Practice Guidelines updated in 2013. Version current 9 April 2013. Internet: http://www.criticalcarenutrition.com/index.php?option=com_content&view=category&layout=blog&id=21&Itemid=10 (accessed 10 December 2013).

36. Yue C, Tian W, Wang W, Huang Q, Zhao R, Zhao Y, Li Q, Li J. The impact of perioperative glutamine-supplemented parenteral nutrition on outcomes of patients undergoing abdominal surgery: a meta-analysis of randomized clinical trials. Am Surg 2013;79:506-13.

Discussion and future perspectives PART I

5

Parts are published in:
Buijs N, Brinkmann SJH, Oosterink JE, Luttikhold J, Schierbeek H, Wisselink W, Beishuizen A, van Goudoever JB, Houdijk APJ, van Leeuwen PAM, Vermeulen MAR.
American Journal of Clinical Nutrition 2015; 101(4): 892-893.

Buijs N, van Leeuwen PAM.
Standard vs. Enriched high protein enteral nutrition in the ICU.
JAMA 2014; 312(21):2288-9.

Buijs N, Vermeulen MAR, van Leeuwen PAM.
Glutamine and Antioxidants in Critically Ill Patients.
New England Journal Medicine 2013; 369(5):484.

The results described in part one of this thesis, concerning the whole-body and interorgan pathway of glutamine, citrulline and arginine, provide new nutritional opportunities to counteract possible disturbances in pathological settings. For the past decades, the intestinal–renal axis, representing this interorgan turnover of glutamine into citrulline and arginine, has received considerable attention from various research groups. The potential positive but also the potential harmful clinical effects of the use of these amino acids in nutritional interventions contributes to the relevance of investigating the role of glutamine as a possible precursor for the synthesis of arginine.

In the general introduction of this thesis, the various mechanisms of action of glutamine, citrulline and arginine are outlined. These so called semi-essential amino acids become essential under conditions of stress when the capacity of endogenous synthesis is exceeded. Just a drop in amino acid levels does not necessarily results in a clinically significant deficiency, however in some catabolic states, biological processes that depend on these semi-essential amino acids are compromised, which may lead to abnormal physiological responses and even poor clinical outcomes. Cancer, but also critical illness, trauma, sepsis and many more catabolic conditions may lead to glutamine and arginine deficiencies (1). Consequently, the effect of enriched nutrition, both enteral and parenteral, with these amino acids is widely studied.

In the 1980s, many randomised trials were performed to investigate the role of glutamine and arginine as a nutritional supplement in metabolically stressed patients. Consistent with the theory, supplementation of these semi-essential amino acids in the deficient states led to improved clinical outcomes. This resulted in adjustments in international nutrition guidelines, recommending enriched nutrition with glutamine and/or arginine in several clinical settings (2,3). However, these recommendations became controversial after the publication of two randomised controlled trials showing possible adverse effects of glutamine supplementation in critically ill patients, the REDOXS trail and the MetaPlus study (4,5). Both study groups investigated the effect of glutamine and antioxidants in a wide range of critically ill patients and found that glutamine supplementation did not have any beneficial effects on ICU mortality and sub-group analyses suggested that it even may be harmful under specific conditions. However, it should be noted that in in the REDOXS trial, glutamine was administered both enteral and parenteral, it was not combined with adequate energy and nitrogen delivery and study doses exceeded the 0.5 $g \cdot kg^{-1} \cdot day^{-1}$ used in previous studies

(including our study described in chapter four). Furthermore, included patients had renal and hepatic dysfunction, which are clear contraindications for glutamine administration (4). In the MetaPlus study however, very low doses of glutamine were provided (< 0.3 $g \cdot kg^{-1} \cdot day^{-1}$) and also in this study they were not able to provide the patients with their required target energy intake (5). Moreover, the results were only found in small subgroup analyses and post-hoc analyses in both studies. Although it is of great importance to take these suggested adverse effects of glutamine supplementation into account in daily practice, given the limitations of these studies more data is needed to clarify the role of glutamine supplementation in various disease states. A meta-analysis recently published by the European Society of Parenteral and Enteral Nutrition showed that glutamine supplementation to critically ill patients according to clinical guidelines as part of an adequate nutritional regimen may significantly improve clinical outcome (6). This indicates that the supplementation of this all-round pharmaco-nutrient should be combined with adequate energy delivery so that the administered glutamine serves as the precursor for important biosynthestic pathways instead of only fuel for depleted cells. Furthermore, it is suggested that several of the effects of glutamine supplementation can be assigned to its precursor role for arginine. Increased arginine availability leads to clinical relevant changes in metabolism. Cancer patients, trauma and surgical patients have been shown to develop disturbances in immune and inflammatory responses and endocrine reactions. This is elaborately outlined in chapter one and two on this thesis. Although some studies show beneficial effects of arginine supplementation in specific patient populations, the latest guidelines of the Society of Critical Care Medicine, the American and European Society of Parenteral and Enteral Nutrition only provide little evidence for immune enhancing nutrition containing arginine in patients undergoing major oncologic surgery, patients with severe trauma and in patients with traumatic brain injury (7-9). In part two of this thesis, the application of arginine enriched nutritional interventions specifically in surgical oncology is further explored. The benefit of arginine administration, mostly combined with other immune enhancing compounds, in surgical cancer patients may be due to its immune stimulating effect on T-lymphocytes and regulation of the circulation through NO. Also, arginine has a positive effect on wound healing. However, arginine administration to patients with sepsis or hemodynamic instability is suggested to cause further deterioration of hemodynamic stability due to vasodilatation and excessive pro-inflammatory responses (10). Insight in metabolic changes and specific nutritional requirements in various disease

states, cancer in particular, could improve the daily care for oncologic patients by development of tailor made nutritional programs.

Studies determining the arterial-venous fluxes of the amino-acids suggested the existence of the intestinal-renal axis of glutamine, citrulline and arginine. These flux studies formed the basis for more in-depth metabolic studies, using stable isotope tracers, which enabled us to investigate the actual conversion of the metabolites on molecular level for the whole-body or specific organs. In chapter four we showed that extra glutamine supplementation in patients undergoing major abdominal surgery results in a promotion of renal arginine *de novo* synthesis from citrulline, compared to patients without a glutamine supplement. This proof-of-concept study is of great value for future research in cancer patients to create new nutritional strategies. Animal studies support that glutamine enriched diets are able to restore host glutamine reserves and support glutamine metabolism, without influencing tumor growth (11). A stable isotope study by Engelen et al. for the first time gave insight in the relation of the diminished arginine availability in cancer patients and endogenous arginine/NO metabolism (12). Interestingly, they showed that the conversion of citrulline into arginine (indicator for NO production) on whole-body level was reduced in patients with advanced non-small cell lung cancer caused by impaired *de novo* arginine synthesis related to a diminished endogenous production of its precursors. An increased whole-body rate of appearance and clearance, which would suggest stimulated arginine breakdown, was not found. They did find that a dietary amino acid mixture (glutamine free) was able to restore arginine to citrulline conversion in the cancer-bearing patients, indicating that correcting arginine availability by a nutritional intervention is able to drive NO synthesis. A more recent study from the same research group showed that plasma arginine, citrulline and glutamine concentration were lower in patients with breast cancer compared to healthy controls, however no differences were found in the rate of appearances of arginine, citrulline and their conversions (13). Surgery in breast cancer patients further reduced the systemic arginine availability due to a combined process of increased arginine breakdown and impaired arginine *de novo* synthesis. In addition, postprandial increase in conversion of citrulline into arginine (indicator for NO production), plasma citrulline and glutamine levels were lower in patients after breast cancer surgery.

Stable isotope methodology to investigate this pathway is still a hot topic in scientific literature and old pitfalls and new strategies come to light. Windmueller and Spaeth were the first to show that both arterial and luminal glutamine are extensively metabolized by the rat gut into CO_2 and other amino acids, in particular,

citrulline (14). In the same study, it was shown that the intestinal production of citrulline accounted for 34% of the glutamine nitrogen turnover, and citrulline accounted for 6% of the glutamine carbon turnover (14,15). Another more recent tracer study in mice showed that the carbon skeleton of glutamine accounted for only a small amount of the carbon found in citrulline, and that glutamine contributed mostly nitrogen to arginine synthesis (16). Whereas tracer studies in humans showed that glutamine does serve as an important carbon and nitrogen precursor for citrulline and arginine (17,18). This raised questions on the precursor relationship of glutamine and citrulline, and subsequently glutamine and arginine.

A strong correlation between glutamine uptake by the small intestine and the release of citrulline has been observed in multiple settings (19-24). Studies showed that plasma concentrations of citrulline and arginine increased after the administration of extra glutamine, and this supports the possible precursor relationship between glutamine, citrulline, and arginine (25-28). However, the increase in plasma levels of citrulline and arginine with provision of glutamine may also be the result of a common element of action: glutamine is the most important fuel for the enterocytes, and citrulline is an indicator of gut mass and function. Therefore, glutamine administration has the potential to augment metabolic activity of the enterocytes and thereby increases citrulline synthesis and levels, without being an actual precursor (29). In addition, since they share important cell membrane transporters, an increase in glutamine availability may cause a reduction in citrulline clearance from the circulation, resulting in higher plasma citrulline concentrations. Though, high correlations between renal arterial citrulline uptake and renal venous arginine release have been observed (30).

When quantifying the metabolic pathway of glutamine to citrulline and arginine with stable isotope methodology, it is important to study the whole-body as well as organ turnover, in order to distinguish the contribution of the intestinal–renal axis and alternative routes. Many studies followed the approach used in the Windmueller and Spaeth publications (14,24,31,32). Stable isotopes allow us to analyze every step within this pathway and, when combined with sampling across organs, even indicate the location of the conversions. Glutamine has been reported to be the main precursor of citrulline in humans, proline in infants and neonates, and arginine in mice. Although this difference may be due to species variations, this may influence the methodological choices in future stable isotope studies. The most frequently used tracer for glutamine has been [2-^{15}N]-glutamine, which was also used in the stable isotope study in this thesis. This glutamine tracer has provided us with a large amount of metabolic data, however during the scientific

quest to unravel the intestinal–renal axis, the application of this tracer to the study of the conversion of glutamine into citrulline recently has become controversial (15-18,33-35). The figure below shows the expected route of the ^{15}N tracer on the amino group (or α-position) from glutamine to citrulline and arginine.

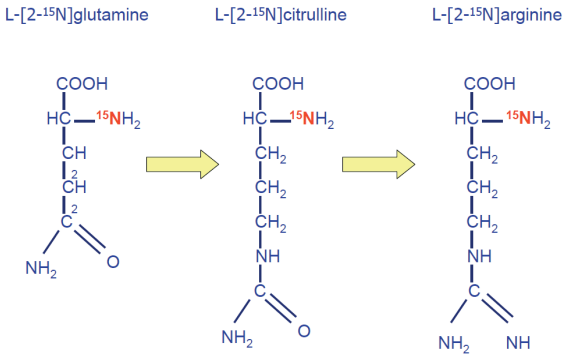

FIGURE 1 | Schematically example of the proposed conversion of the tracer [2-^{15}N]glutamine tracer into [2-^{15}N]citrulline and [2-^{15}N]arginine.

As shown in Figure 1, there is a nitrogen molecule in glutamine in both the α-position and the δ-position or amido group. The citrulline molecule has nitrogen molecules at three locations: the α-, δ-, and ureido position. It has been shown that the ^{15}N of the glutamine tracer does not always remain in the original position during the conversion into citrulline, since it is also retrieved from other positions on the citrulline molecule (16,36). The enrichment of citrulline is usually determined by measuring the abundance of the marked ions, as in our study, and this may lead to overestimating the contribution of the carbon skeleton of glutamine to citrulline synthesis. Simultaneous infusion of an ^{15}N glutamine tracer and a citrulline tracer (usually a carbon-labeled tracer, such as [^{13}C]-citrulline) results in a larger amount of glutamine converted into arginine, compared to the conversion of citrulline into arginine. This was also seen in our stable isotope study and this implies that the use of a nitrogen tracer does indeed overestimate the conversion that occurs in the essential intermediate step. This also suggests that alternative routes, for example, transamination, nitrogen recycling, or channeling, contribute to the incorporation of the labeled nitrogen tracer into citrulline and arginine.

Using tandem mass spectrometry techniques (LC-MS/MS) in the multireacting mode (MRM), allows specific fragmentation of the analytes of interest (36).

This makes it possible to detect the location of a tracer within the molecule. In combination with the fact that nitrogen tracers may appear in different locations on the product molecule, this method could give us more information on the exact conversion of glutamine or parts of its molecule into other molecules, including citrulline and arginine. Another recently developed technique using an Orbitrap mass detector enables separation of the isotopes of C, N, D, and O in a single compound by using high-resolution mass spectrometry. This technique makes it possible to distinguish between multiple isotopic labels within a compound (37).

Future perspectives

More high-quality studies are needed to investigate the exact clinical effect of glutamine and arginine supplementation. I would suggest a well-designed randomized controlled trial in cancer patients. When determining amino acid metabolism before and after a nutritional intervention and combine these results with clinical outcome data, specific metabolic disturbances could be associated with decreased outcome measures. This could be a future opportunity to screen specific patients on their metabolic status and subsequently prescribe them a tailor made nutritional program. Furthermore, up-to-date metabolic requirement tests could adjust nutritional intervention on demand.

It is clear that more research is needed to elucidate the complex pathway of the intestinal-renal axis. The implementation of new tracer techniques could be of great value to improve our understanding of the intestinal-renal axis in several disease states. New approaches may change our whole idea of the glutamine, citrulline, and arginine pathway as it is now: a complex pathway that still needs unraveling. Researchers in this field should be aware of the possible alternative metabolic routes of the infused tracers and should be informed of the correct approach to analyzing those tracers to allow adequate determination of the amino acid metabolism. Stable isotope research on the intestinal–renal axis may present big challenges in clinical practice, cost, and effort. However, tracer studies can teach us a lot about the in-depth conversions of this complex pathway, and they also may improve clinical outcomes by providing important information for the development of tailor-made nutrition for specific patient populations. Future studies are necessary to further determine the complicated interorgan route of glutamine, citrulline and arginine in the cancer-bearing state.

References

1. Morris CR, Hamilton-Reeves J, Martindale RG et al. Acquired Amino Acid Deficiencies: A Focus on Arginine and Glutamine. Nutr Clin Pract. 2017 Apr;32(1_suppl):30S-47S.
2. Singer P, Berger MM, van den Berghe BG et al. ESPEN guidelines on parenteral nutrition: intensive care. Clin Nutr. 2009; 28: 387–400
3. McClave SA, Martindale RG, Vanek VW et al. Guidelines for provision and assessment of nutritional support therapy in adult critically ill patient: society of Critical Care Medicine (SCCM) and American Society for Parenteral Nutrition (A.S.P.E.N.). J Parenter Enteral Nutr. 2009; 33: 277–31
4. Heyland D, Muscedere J, Wischmeyer PE et al. A randomized trial of glutamine and antioxidants in critically ill patients. N Engl J Med 2013;368(16):1489-1497.
5. van Zanten AR, Sztark F, Kaisers UX et al. High-protein enteral nutrition enriched with immune-modulating nutrients vs standard high-protein enteral nutrition and nosocomial infections in the ICU: a randomized clinical trial. JAMA. 2014;312(5):514-24.
6. Stehle P, Ellger B, Kojic D et al. Glutamine dipeptide-supplemented parenteral nutrition improves the clinical outcomes of critically ill patients: a systematic evaluation of randomised controlled trials. Clin Nutr ESPEN 2017;17:75-85.
7. McClave SA, Taylor BE, Martindale RG et al. Guidelines for the provision and assessment of nutrition support therapy in the adult critically ill patient: Society of Critical Care Medicine (SCCM) and American Society for Parenteral and Enteral Nutrition (A.S.P.E.N.). JPEN J Parenter Enteral Nutr. 2016;40(2):159-211.
8. Weiman A, Braga M, Carli F et al. ESPEN Guideline: Clincal nutrition in surgery. Clin Nutr 2017;36:623-650.
9. Arends J, Bachmann P, Baracos V et al. ESPEN guidelines on nutrition in cancer patients. Clin Nutr 2017;36(1):11-48.
10. Patel JJ, Miller KR, Rosenthal C et al. When is it appropriate to use arginine in critical illness? Nutr Clin Pract 2016;31(4):438-44.
11. Klimberg VS, Souba WW, Sailoum RM, et al. Glutamine-enriched diets support muscle glutamine metabolism without stimulating tumor growth. J Surg Res 1990;48(4):319-323.
12. Engelen MP, Safar AM, Bartter T, Koeman F, Deutz NE. Reduced arginine availability and nitric oxide synthesis in cancer is related to impaired endogenous arginine synthesis. Clin Sci (Lond) 2016;130(14):1185-1195.
13. Engelen MP, Klimberg VS, Allasia A, Deutz NE. Major surgery diminishes systemic arginine availability and suppresses nitric oxide response to feeding in patients with early stage breast cancer. Clin Nutr 2017;36(5): S0261-5614(17)30260-1.
14. Windmueller HG, Spaeth AE. Uptake and metabolism of plasma glutamine by the small intestine. J Biol Chem 1974;249(16):5070-5079.
15. Marini JC. Interrelationships between glutamine and citrulline metabolism. Curr Opin Clin Nutr Metab Care 2016;19(1):62-66.
16. Marini JC, Didelija IC, Castillo L, Lee B. Glutamine: precursor or nitrogen donor for citrulline synthesis? Am J Physiol Endocrinol Metab 2010;299(1):E69-E79.
17. Kao C, Hsu J, Bandi V, Jahoor F. Alterations in glutamine metabolism and its conversion to citrulline in sepsis. Am J Physiol Endocrinol Metab 2013;304(12):E1359-E1364.
18. Tomlinson C, Rafii M, Ball RO, Pencharz P. Arginine synthesis from enteral glutamine in healthy adults in the fed state. Am J Physiol Endocrinol Metab 2011;301(2):E267-E273.
19. Boelens PG, van Leeuwen PA, Dejong CH, Deutz NE. Intestinal renal metabolism of L-citrulline and L-arginine following enteral or parenteral infusion of L-alanyl-L-[2,15N] glutamine or L-[2,15N]glutamine in mice. Am J Physiol Gastrointest Liver Physiol 2005;289(4):G679-G685.

20. Peters JH, Wierdsma NJ, Teerlink T, van Leeuwen PA, Mulder CJ, van Bodegraven AA. The citrulline generation test: proposal for a new enterocyte function test. Aliment Pharmacol Ther 2008;27(12):1300-1310.
21. Souba WW, Herskowitz K, Salloum RM, Chen MK, Austgen TR. Gut glutamine metabolism. JPEN J Parenter Enteral Nutr 1990;14(4 Suppl):45S-50S.
22. van de Poll MC, Ligthart-Melis GC, Boelens PG, Deutz NE, van Leeuwen PA, Dejong CH. Intestinal and hepatic metabolism of glutamine and citrulline in humans. J Physiol 2007;581(Pt 2):819-827.
23. van de Poll MC, Siroen MP, van Leeuwen PA et al. Interorgan amino acid exchange in humans: consequences for arginine and citrulline metabolism. Am J Clin Nutr 2007;85(1):167-172.
24. Windmueller HG, Spaeth AE. Source and fate of circulating citrulline. Am J Physiol 1981;241(6):E473-E480.
25. Boelens PG, Melis GC, van Leeuwen PA, ten Have GA, Deutz NE. Route of administration (enteral or parenteral) affects the contribution of L-glutamine to *de novo* L-arginine synthesis in mice: a stable-isotope study. Am J Physiol Endocrinol Metab 2006;291(4):E683-E690.
26. Houdijk AP, van Leeuwen PA, Teerlink T, et al. Glutamine-enriched enteral diet increases renal arginine production. JPEN J Parenter Enteral Nutr. 1994;18(5):422-426.
27. Ligthart-Melis GC, van de Poll MC, Dejong CH, Boelens PG, Deutz NE, van Leeuwen PA. The route of administration (enteral or parenteral) affects the conversion of isotopically labeled L-[2-15N]glutamine into citrulline and arginine in humans. JPEN J Parenter Enteral Nutr 2007;31(5):343-348.
28. Ligthart-Melis GC, van de Poll MC, Vermeulen MA et al. Enteral administration of alanyl-[2-(15)N]glutamine contributes more to the *de novo* synthesis of arginine than does intravenous infusion of the dipeptide in humans. Am J Clin Nutr 2009;90(1):95-105.
29. Beaufrere AM, Neveux N, Patureau MP et al. Long-term intermittent glutamine supplementation repairs intestinal damage (structure and functional mass) with advanced age: assessment with plasma citrulline in a rodent model. J Nutr Health Aging 2014;18(9):814-819.
30. Deutz NE. The 2007 ESPEN Sir David Cuthbertson Lecture: amino acids between and within organs. The glutamate-glutamine-citrulline-arginine pathway. Clin Nutr 2008;27(3):321-327.
31. Windmueller HG, Spaeth AE. Intestinal metabolism of glutamine and glutamate from the lumen as compared to glutamine from blood. Arch Biochem Biophys 1975;171(2):662-672.
32. Windmueller HG, Spaeth AE. Respiratory fuels and nitrogen metabolism *in vivo* in small intestine of fed rats. Quantitative importance of glutamine, glutamate, and aspartate. J Biol Chem 1980;255(1):107-112.
33. Ligthart-Melis GC, Vermeulen MA, van Leeuwen PA, Deutz NE. Glutamine: precursor or nitrogen donor for citrulline synthesis? Am J Physiol Endocrinol Metab 2010;299(4):E683.
34. Ligthart-Melis GC, Deutz NE. Is glutamine still an important precursor of citrulline? Am J Physiol Endocrinol Metab 2011;301(2):E264-E266.
35. Ligthart-Melis GC, Marini JC, Engelen MP, Deutz NE. Glutamine supplementation, citrulline production, and *de novo* arginine synthesis: is there a relation? Am J Clin Nutr 2015;101(4):890-892.
36. Marini JC. Quantitative analysis of 15N-labeled positional isomers of glutamine and citrulline via electrospray ionization tandem mass spectrometry of their dansyl derivatives. Rapid Commun Mass Spectrom 2011;25(9):1291-1296.
37. Frey AJ, Feldman DR, Trefely S, Worth AJ, Basu SS, Snyder NW. LC-quadrupole/Orbitrap high-resolution mass spectrometry enables stable isotope-resolved simultaneous quantification and (1)(3)C-isotopic labeling of acyl-coenzyme A thioesters. Anal Bioanal Chem 2016;408(13):3651-3658.

PART II

ARGININE/NO METABOLISM IN SURGIGAL ONCOLOGY

The role of a disturbed arginine/NO metabolism in the onset of cancer cachexia: A working hypothesis

Buijs N
Luttikhold J
Houdijk APJ
Van Leeuwen PAM

Published in:
Current Medical Chemistry 2012; 19: 5278-5286.

abstract

Cancer cachexia is a complex catabolic state in patients with a malignancy, associated with increased morbidity and mortality. This syndrome is characterized by a redistribution of the body's protein content and a subsequent muscle wasting. The etiology of this syndrome seems multifactorial, but remains unclear. It is suggested that this catabolic state occurs in response to the alterations in immune interactions between tumor and host. The amino acid arginine and its derivate nitric oxide (NO) play various roles in anti-tumor immune response and the body's homeostasis. Glutamine is the precursor for arginine *de novo* synthesis and the most abundant amino acid in the body, mainly stored in skeletal muscle. Tumors develop a protection mechanism against the specific anti-tumor attack of the immune system by recruiting myeloid-derived suppressor cells (MDSC). The MDSC deplete arginine levels and disturb NO production.

We here hypothesize that the perturbation of the arginine/NO metabolism plays a significant role in the etiology of cancer cachexia. Arginine/NO metabolism is disturbed in patients with cancer. The body will try to correct this perturbation by mobilizing arginine and glutamine from muscles. The decreased arginine levels and the disturbed NO production activate several cascades, which in turn inhibit protein synthesis and promote proteolysis, leading to cachexia. Cachexia remains one of the most frequent and damaging opportunistic syndromes in cancer patients. In this review, we will elaborate on a new hypothesized concept and the underlying mechanisms of this syndrome. New studies are essential to ground this hypothesis and to develop interventions to break through the pathological mechanisms underlying cachexia.

Introduction

The designation cachexia is derived from the Greek words *kakos* (bad) and *hexis* (condition). The international accepted definition of cachexia is "a complex metabolic syndrome associated with underlying illness and characterized by loss of muscle with or without loss of fat mass" (1). Weight loss is observed in up to 80 percent of all cancer patients and it is associated with a reduced survival rate (2,3). Almost one third of all cancer patients die from cachexia rather than from cancer itself (4). Most obvious in this syndrome is the elimination of the body's protein content. Cancer cachexia is characterized by an ongoing loss of skeletal muscle mass, which is the result either from a decrease in protein synthesis, an increase in protein degradation, or a combination of both (5). The etiology of cancer cachexia seems multifactorial, but remains unclear. Tumor and host-derived factors cause multiple alterations in immune response and inflammation resulting in this catabolic state.

The body's immune system is one of the most important factors that may prevent the evolvement of a malignant tumor. However, in the presence of a malignancy the immune system is disarmed by tumor-derived mechanisms. Inflammation and the subsequent production of pro-inflammatory mediators are strongly associated with cancer. In the first phase of the carcinogenesis, at the onset of malignant initiation, this inflammation is the result of the normal protective mechanism of the host to combat pathological evolving cells. However, though these processes are self-limiting in general, dysregulation of this response can lead to the initiation or progression of the (pre)malignant disease. A perturbation in the expression of pro-inflammatory mediators augments tumor promotion by the induction of factors that support angiogenesis, tumor cell growth and tissue emodeling. In addition, these pro-inflammatory mediators are the initiators for the inhibition of the immune response by T-lymphocytes, Natural Killer (NK) cells, macrophages and neutrophils against the tumor. Moreover, tumor-derived inflammatory cells migrate into the tumoral stroma and various immune involved organs, and on their turn disbalance the inflammatory reaction and anti-cancer immune response. These alterations may induce tumor promoting inflammation, suppression of the immune system and tumor progression. Experts in the field of nutrition in oncology concur that cancer cachexia may be a result of an interaction between this pathological inflammation and immune suppression (6).

In the last decades, the semi-essential amino acid arginine and its derivate NO have been shown to play major roles in different homeostatic and immunological processes. The arginine/NO metabolism is disturbed in various diseases, including cancer, and this contributes to the alterations in immune function and metabolism. We propose that arginine may play a major role in the onset of cachexia. In this review, we will clarify our hypothesis, which suggests that a disturbed arginine/NO metabolism in cancer patients may contribute to the initiation and progression of cancer cachexia. With this knowledge, we may be able to optimize our nutritional interventions to diminish the negative consequences of cachexia.

The arginine deficiency – cancer cachexia hypothesis

We propose that the perturbation in the arginine/NO pathway in cancer patients plays a causal role in the development of the cachexia process. To overcome the arginine deficiency, present in cancer patients, this pathway may adapt in the already identified catabolic mechanisms by compromising the immune system and disorganizing the regulation of protein turnover.

The current perspectives

The identified pathogenesis of cancer cachexia

Cancer cachexia is characterized by a depletion of skeletal muscle, with a 75% decrease in protein content at 30% body weight loss, whereas the non-muscle protein compartment is relatively preserved (7). Several tumor and/or host induced factors are proposed to mediate this malignant catabolism by inhibiting protein synthesis and promoting protein breakdown.

Protein Synthesis. Protein synthesis in skeletal muscle is primarily regulated at the initiation phase of protein translation, a highly complex and conserved process with numerous mediators involved (8). There are two points of control in this process. The first is the binding of the initiator methionyl tRNA (mettRNA) to the 40S ribosomal subunit. This is regulated by eukaryotic initiation factor 2 (eIF2) and mediated by guanosine triphosphate (GTP). eIF2 binds to GTP, and the ternary complex eIF2.GTP.met-tRNA binds to the 40S ribosomal subunit, together with eIF3 forming the 43S pre-initiation complex. Once the initiation

is completed, eIF2 is released from the ribosome and bound to the inactive guanosine diphosphate (GDP). To mediate in the next cycle of translation initiation GDP must be exchanged for GTP. This recycling process of GTP can be inhibited by phosphorylation of eIF2 on the α-unit by eIF2α kinases, which respond to stress conditions that affect transcription and protein synthesis. This subsequently inhibits the initiation of protein translation. Although some studies suggest that decreased protein synthesis in skeletal muscle is the result of anorexia, protein synthesis is also depressed in cachexia where anorexia is absent, which suggests an underlying defect in protein synthesis regulation. In cachectic cancer patients, an increase in eIF2α kinase activity is described and a subsequent increase in phosphorylation of eIF2α, hereby inhibiting myofibrillar protein synthesis. Furthermore, it is found that when the activity of eIF2α kinase is inhibited, the phosphorylation of eIF2α decreases, which enables the conversion of eIF2.GDP to eIF2.GTP and thus reverse the inhibition of protein synthesis (9). Moreover, stimulation of the eIF2α kinases have been found to reduce protein synthesis and also activate various transcription factors which, in turn, induce the transcription of genes necessary for amino acid synthesis and transport (10). Another crucial step in the process of protein translation involves the formation the active eIF4F complex. This complex consists of three subunits eIF4E, eIF4A and eIF4G. The main regulatory action is the conversion of the inactive complex eukaryotic initiation factor 4E (eIF4E) and its binding protein (4EBP1) to the active eIF4G-eIF4E complex, mediated by phosphorylation of 4E-BP1 and mammalian target of rapamycin (mTOR) (11). Weight loss in tumor-bearing mice is associated with an increased amount of the inactive eIF4E-4E-BP1 complex in muscle, due to hypophosphorylation of 4E-BP1, as well as a decrease in phosphorylated mTOR, resulting in inhibition of translation initiation (12). To reverse the inhibition, mTOR has to be activated to cause hyperphosphorylation of 4E-BP1, resulting in the release of eIF4E to form the active eIF4F complex (8).

Thus, these two points off control are the regulating steps in protein synthesis. A disturbance in these steps will result in a dysregulation of protein synthesis.

Protein Breakdown. Furthermore, increased protein breakdown may also result in cachexia related muscle wasting. The most important pathway regulating myofibrillar protein degradation in cachexia is the ubiquitinproteasome pathway, which consists of two main steps: the ubiquitination of protein and the degradation of ubiquitanated protein by 26S proteasoma (13). In the first step, E1 Ub-activating enzyme, E2 Ub-carrier protein and E3 Ub-protein ligase mediate the ubiquitination

of the muscle proteins. Atrogin-1 is a muscle-specific F-box type E3 ligase that is induced 8 to 40-fold in muscle atrophy during cancer (14). MuRF1 is a ring finger type muscle-specific E3 ligase and plays an important role in myofibrillar protein breakdown. Both muscle-specific E3 ligases are considered to be essential in muscle protein catabolism, because knockout mice lacking these E3 ligases are prevented from muscle wasting (15). Moreover, besides their role in translation regulation, the eIF2α kinases activate the transcription of nuclear factor kappa B (NFκB), a regulator of DNA transcription, leading to an increased expression of the ubiquitinproteasome pathway (16). As a result of this activation of protein breakdown, ammonia production is increased and subsequently the formation of urea is stimulated (17). Especially the protein myosin heavy chain, the principal muscle contractile protein, is selectively eliminated by the ubiquitin-proteasome pathway in cachectic muscle wasting, while other myofibrillar proteins are preserved (18).

Catabolic Mediators. As stated in the introduction, a disturbance in the host immune response in the presence of a malignancy causes a pathological attendance of immune cells and production of signaling factors. Several tumor and host-derived factors have been implicated in the pathogenesis of cancer cachexia. Pro-inflammatory cytokines are involved in the metabolic disturbances in cancer cachexia. These cytokines can be produced by the tumor, tumor infiltrating immune cells and other cells. Although there is no consensus for the exclusive roles of any of these cytokines, several of them have been shown to evidently mediate the cachectic process (19,20). Various studies showed that the cytokines tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) are major triggers of muscle wasting. An increase in TNF-α levels is associated with a decline in muscle mass and strength (21). TNF-α mediates its effects on muscle protein turnover by activation of NFκB. There are three main mechanisms by which NFκB induces muscle wasting. The first is the inhibition of protein synthesis, the second is the upregulation of the ubiquitin-proteasome pathway and the last is the induction of oxidative stress in muscle cells (22-24).

Protein synthesis is inhibited by decreasing the formation of the active eIF4F complex by hypophosphorylation of the binding protein 4E-BP1 (25). Furthermore, TNF-α may cause degradation of muscle protein synthesis by the degradation of MyoD, a myogenic transcription factor promoting muscle fiber formation. The loss of MyoD leads to a reduction in myosin heavy chain (MyHC) expression (26). Increased levels of TNF-α induce loss of Jun-D activity in muscle tissue. The decrease of this transcription factor causes a decrease in

muscle creatine kinase (CKM) expression. This enzyme regulates ATP reserves and muscle function. Therefore, a depletion of CKM provides a molecular impulse for the onset of muscle wasting (23). It has been shown that the effects of TNF-α are more pronounced when also IFN-γ is expressed (26). TNF-α has been shown to upregulate the ubiquitin-proteasome pathway through an increase in expression of various ubiquitin transcripts and the ubiquitin ligase atrogin-1 (24,27). Also, the oxidative stress, initiated by TNF-α, directly induces skeletal muscle protein loss by stimulation of the ubiquitin-proteasome pathway. Furthermore, oxidative stress may cause direct apoptosis in muscle cells.

Moreover, several cytokines from the interleukin family, like IL-6 and IL-8, have been shown to play a promoting role in cancer related muscle wasting, while other cytokines of this family, like IL-4, seem to have a preventive function (28). The actions of the pro-cachectic factors described seem to be indirect and the actual catabolic effects are proposed to be caused by more direct mediators.

Support of hypothesis

Cancer, a condition associated with a perturbation in arginine/NO metabolism

Arginine is a semi-essential amino acid. This means that in physiological conditions exogenous and endogenous arginine meet the needs of the adult human body. However, during growth or disease, the body requires more arginine than present and arginine *de novo* synthesis is inadequate. As defined in the introduction, malignant tumors may cause immune suppression in the host. It has been shown that cancer interferes with arginine metabolism to combat the immune responses (29). Reduction of extracellular arginine levels generates an arrest in the proliferation of activated T-cells and blocks the re-expression of the CD3ζ chain (30). Furthermore, in the absence of arginine, T-cells are arrested in the G0-G1 phase of the cell cycle and can not proliferate because of a cyclin D deficiency. T-cells activated in an arginine free environment will develop all the alterations as found in cancer patients and tumor-bearing mice (31). Furthermore, NO is used in the immune response by cytotoxic cells and many other cells of the immune system. A disturbance in the NO production also results in the blockage of T-cells proliferation and reactive nitrogen species may cause a cell cycle arrest (32).

Cancer and the subsequent chronic inflammation create a protection mechanism against the specific anti-tumor immune response of the host. One of the most important immune suppressing strategies is the inflow of innate immature myeloid cells. Primarily, these cells are recruited to combat the malignant tumor, however because of a disbalance in pro-inflammatory mediators, cytokines and chemokines, these immature cells develop into a tumor promoting phenotype (33). Myeloid-derived suppressor cells (MDSC) accumulate in the tumor environment, lymph nodes, spleen and liver of the tumor-bearing host. They represent a heterogeneous population of immunosuppressive cells expressing a variety of surface markers. These MDSC produce the two most important enzymes involved in arginine catabolism: arginase I and inducible nitric oxide synthase (iNOS) (34,35).

Arginase I metabolizes arginine into urea and ornithine (36). The arginase activity is increased by 6-10 fold in cancer patients (37). Arginase I is the only arginine metabolizing enzyme able to modify serum levels of arginine (38). Increased arginase activity induces a depletion of extracellular arginine and increases the uptake of arginine trough the y+ system of cationic amino acid transporters (CAT). Accordingly, arginine concentrations are decreased in patients with cancer (39). Alternatively, arginine is converted by iNOS into NO, which has many important cell signaling roles in the immune function and protein turnover. NO is an important vasodilator and regulator of the blood flow. Furthermore, NO can be generated by immune cells which use NO for cytostatic immune responses and cell proliferation (40). Under conditions of limiting arginine, when both arginase I and iNOS are induced, reactive nitrogen species are generated by iNOS. These highly reactive oxidizing agents may cause apoptosis in activated T-cells and have many more inhibiting and destructive effects on a variety of cell types (41).

In physiological conditions, the majority of the arginine *de novo* synthesis involves the "intestinal-renal axis": glutamine is converted into citrulline in the small intestine. Citrulline is mainly produced by enterocytes without incorporation into proteins. Subsequently, citrulline is metabolized in the kidney into arginine (42). Glutamine is the most abundant amino acid in the body with skeletal muscles as most important reservoir. Glutamine is consumed by cells with rapid turnover and participates in regulation of protein synthesis. The glutamine levels in skeletal muscle correlate with muscle protein mass (43).

Citrulline is a non-protein amino acid, which is very efficiently transported into enterocytes and generally escapes hepatic uptake. Therefore, citrulline is a very

important metabolic intermediate (44). The conversion of citrulline into arginine is the only way for citrulline utilization inside the cells. Healthy humans with functional kidneys have high rates of arginine synthesis from endogenous and exogenous citrulline (45). After proceeding the intestinal-renal axis, arginine is used in the immune system for the proliferation of T-cells, functions as the precursor for NO, regulates the urea cycle and has numerous roles in other homeostatic systems.

However, in the presence of a malignancy, arginase and iNOS activity are increased on the whole-body level, essentially caused by the MDSC (46). This induces a decrease in plasma arginine concentration, a pathologic production of NO and the formation of reactive nitrogen species, like peroxynitrite. Moreover, exogenous arginine and the arginine *de novo* synthesis fail to meet the high demand for arginine. Accordingly, we propose that the perturbations in the arginine/NO pathway will initiate a correction of this harmful disturbance. Skeletal muscle consists for a major part of glutamine, the substrate for arginine *de novo* synthesis. To correct the arginine deficiency, glutamine may be recruited from skeletal muscles and this redistribution of the protein content results in muscle wasting. Muscle wasting is the result of depression in protein synthesis, an increase in proteolysis, or a combination of both. When arginine levels are low, protein synthesis will attenuate to preserve the already deficit arginine levels. Furthermore, increased protein breakdown will generate the release of arginine and its precursor glutamine from the muscles.

One of the most important characteristics of muscle wasting in cachexia is that, different from starvation induced wasting, it cannot be restored by conventional nutritional supplements (5). This supports the indication that complex metabolic changes underlie the onset of this catabolic state (Figure 1).

Arginine deficiency induced modification of the protein turnover in cancer

To correct the arginine deficiency, arginine and its precursors have to be preserved. To establish this, the translation initiation and protein degradation machinery adapts to the requirements in times of an arginine deficiency. Also, the increased NO levels, caused by the increased iNOS expression, effect the process of general proteolysis and protein synthesis. This disturbance in the arginine/NO metabolism has been shown to interfere in the most crucial steps in these processes, seen in cancer cachexia.

Protein Synthesis. When arginine levels are low the demand for arginine by protein synthesis has to be reduced. In an arginine deficiency state eIF2α kinases are stimulated, leading to phosphorylation of eIF2α (47,48) and subsequently to the inhibition of muscle protein synthesis. Furthermore, the increased iNOS activity in the tumor-bearing host also activates the eIF2α kinases and thus inhibits protein synthesis (49).

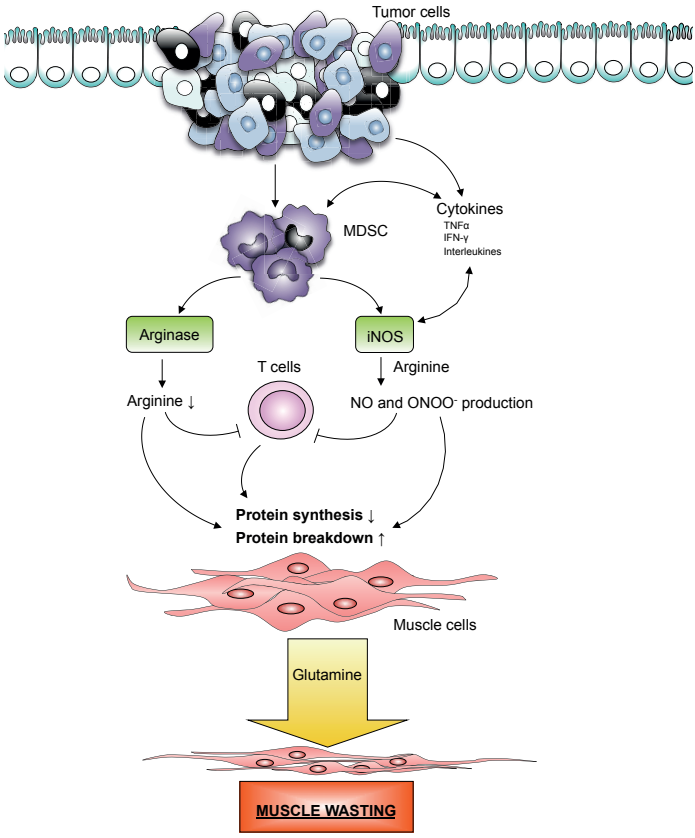

FIGURE 1 | The hypothesized role of the disturbed arginine/NO pathway in the onset and development of cancer cachexia.

In the second regulatory step in the translation initiation, adequate arginine levels are required for the phosphorylation of mTOR and the subsequent phosphorylation of 4E-BP1 in muscle. Also, the increased urea synthesis leads to

activation of the mTor pathway (50). This promotes the formation of the eIF4F complex and thus enhances protein synthesis (51,52). However, one can suggest that when arginine levels are decreased the translation initiation will stagnate at this important regulatory step. Another study found that increased NO levels inhibit the mTOR pathway as well and induce the phosphorylation of initiation factors of protein translation, like eIF2α, leading to degradation of general protein synthesis (53,54).

When arginine is depleted by high arginase I activity, NO reacts with the simultaneously produced superoxide to form peroxynitrite (ONOO-). This reactive and toxic molecule induces oxidative stress in muscle cells (55,56), which is illustrated by increased levels of malondialdehyde, a degenerative product of oxidative stress, in skeletal muscle of cachectic cancer patients. Peroxynitrite also has been shown to activate eIF2α kinases and thus inhibit protein synthesis (48). Furthermore, peroxynitrite selectively inhibits MyoD, leading to a reduction in MyHC expression and thus compromising the integrity of the myofibrillar protein complex (Figure 2).

Protein Breakdown. Moreover, evidence shows that adequate arginine levels are essential not only for protein synthesis, but also for the regulation of protein breakdown in skeletal muscle (57). Arginine is found to decrease the ubiquitin protein kinases atrogin-1 and MuRF1 and thus to decrease protein degradation. It is assumed that an arginine deficiency causes an over expression of both E3 ligases and leads to muscle atrophy. Although the exact mechanism of the inhibitory effect of arginine on MuRF1 remains unclear, the decrease in atrogin-1 expression is dependent of the phosphorylation of mTOR (58). Therefore, adequate regulation of the arginine levels may improve protein turnover and generate muscle gain (59).

Furthermore, high peroxynitrite levels in cachexia are associated with increased expression of several proteolytic systems, such as the increased expression and activity of the intracellular ubiquitin-proteasome pathway (60,61). Peroxynitrite causes a prolonged alternative activation of the NF-κB complex, this pleyotropic transcription factor participates in several muscle wasting processes. In the presence of peroxynitrite increased levels of ubiquinated MyHC are found, which indicates an activation of the ubiquitin-proteasome pathway, mediated by NF-κB (62,63). To support the previous clarification on the role of iNOS and NO in muscle wasting, Buck et al. described that muscle wasting in cachexia can be prevented by inhibitors of iNOS (23)(Figure 2).

Catabolic Mediators. Arginine and its derivate NO are known to have many regulatory roles, and also in cancer cachexia they have various points of action. Decreased arginine levels are associated with the stimulation of the expression of several pro-cachectic cytokines, such as TNF-α, IL-6 and IL-8 (64,65). The increased iNOS activity in cancer patients causes pathologic NO levels and several NO dependent pathways may be responsible for the induction of muscle wasting (66). As described before, cytokines seem to be one of the main causative factors in the development of cancer cachexia (8). Studies showed that the cytokines TNF-α and IFN-γ are major triggers of muscle wasting (67). These cytokines are a stimulus to increase iNOS expression and thus promote the conversion of arginine into NO. TNF-α activates the NF-κB complex, which enhances the transcription of iNOS (55). It seems that many of the effects of TNF-α are mediated by NO. There is a consistent increase in TNF-α and iNOS levels in patients with cancer cachexia, which indicates that iNOS and increased NO levels mediate in the effects of TNF-α in muscle wasting (68). Furthermore, TNF-α promotes NO production, by increasing the arginine uptake of the cells trough the y+system of the CATs, and thus decrease arginine levels even more (69). TNF-α stimulates the membrane-bound NADPH oxidase complex, which also results in the release of superoxide ($O2^{-}$) and causes formation of peroxynitrite (70). Furthermore, the loss of Jun-D activity in muscle tissue, induced by increased levels of TNF-α is shown to be mediated by the upregulation of iNOS. As stated before, this disturbance provides a molecular impulse for the onset of muscle wasting. IFN-γ was found to potentiate the effects of TNF-α on this disturbed iNOS/NO balance (71,72). Other studies showed that arginine is able to decrease interleukins levels that promote muscle wasting, like IL-6 (73).

Reflexion in whole-body arginine metabolism in cancer patients

As described above, cells have the capacity to detect alterations in the availability of specific nutrients and alter the expression of key genes necessary to adapt under new conditions. This is a perfectly regulated mechanism in healthy subjects. However, in tumor-bearing subjects these alterations in protein turnover are reflected in a perturbation of the amino acid metabolism on whole-body level. This further supports our hypothesis.

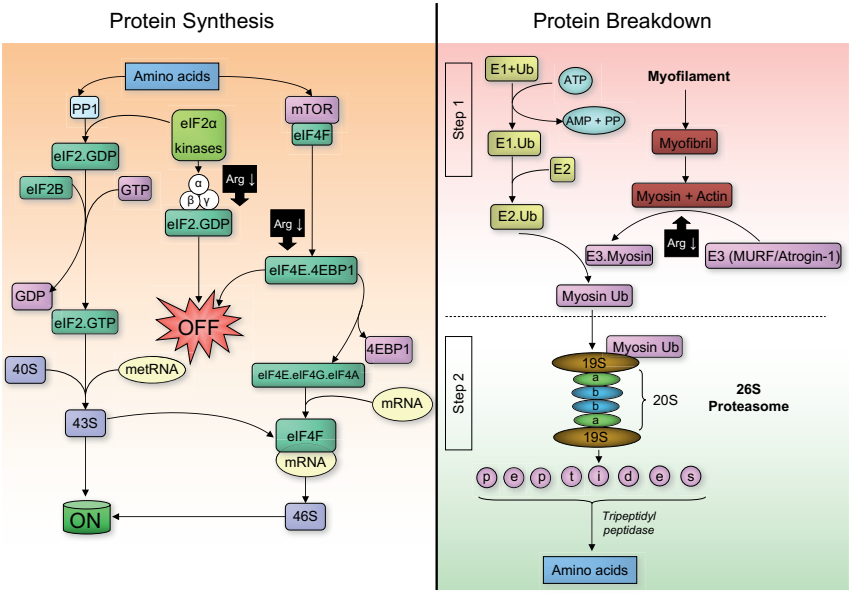

FIGURE 2 | The mechanisms of protein synthesis and protein breakdown. The boxes with "Arg ↓" show the points of impact of the disturbed arginine metabolism in cancer patients.

In the arginine deficiency state, an increased muscle glutamine output is found, accompanied with a decreased intracellular glutamine concentration (74,75). Tumor growth is inversely related to host glutamine stores, and cancer cachexia is marked by massive host skeletal muscle glutamine depletion (76). Several studies show an increase in protein breakdown in the presence of a small tumor, with a concomitant increase in muscle glutamine production, but depletion in plasma glutamine levels. In rats bearing larger tumors whole-body glutamine turnover increased, though muscle glutamine turnover decreased, likely because of the depleted muscle glutamine pool. The increase in whole-body glutamine turnover appears to be caused by increased turnover in cells of the immune system and visceral organs (17,77). During cancer the glutamine uptake by the gut and the conversion into citrulline is increased (75). However, in this condition the liver starts to utilize citrulline, hereby compromising arginine *de novo* synthesis. The hepatic citrulline uptake limits the amount of gut-derived citrulline reaching the kidney, so less citrulline is taken up by the kidneys and renal arginine production is decreased (78). At the same time the hepatic arginase I activity and the urea production are higher and consequently the arginine utilization in the liver is increased (75,79,80).

This might be explained by two mechanisms: during a hypercatabolic state, like cancer cachexia, ammonia levels increase because of high amino acid breakdown. Arginine is necessary to wash out ammonia in the urea cycle and hereby prevents the pathological effects of hyperammonemia (17). Complementary, the increased arginine utilization in the liver may be a result of the inflow of the arginase and iNOS producing MDSC into the liver in the cancer bearing host (81). Thus, arginine levels remain low. Reduced arginine levels, caused by arginase, are the drive for arginine production by the kidney in non-tumor-bearing rats (82), but apparently, there is a shift in the intestinal-renal axis metabolism in the presence of a malignancy: *de novo* arginine production is decreased in tumor-bearing hosts compared with controls, despite of the arginine deficiency (Figure 3).

Another plausible explanation of the disturbance in the arginine metabolism may be the consumption of arginine and its precursors by the compromised immune system. In an arginine deficient environment, T-cells develop a compensating mechanism by using citrulline as a substrate too. Citrulline is transported across the cell membrane, into T-cells, by a specific membrane transport protein. Now intracellular, citrulline is metabolized by argininosuccinate synthetase (ASS) to citrullyl-AMP, which is further metabolized by argininosuccinate lyase (ASL) into arginine (83). Up-regulation of ASS expression has been described in several cell lines in an arginine deficiency state (84-86). The T-cell-derived arginine is directly used for T-cell proliferation and in this way escapes the high arginase activity. Unfortunately, this physiological compensating mechanism does not suffice the high arginine demand and T-cell proliferation remains inadequate in these patients. Furthermore, by producing their own arginine, T-cells withhold the kidneys from citrulline for arginine *de novo* synthesis and therefore stimulate proteolysis and decreased protein synthesis even more (Figure 3).

The adjustments in protein synthesis and protein breakdown to compensate the arginine depletion cause increased glutamine output in skeletal muscle, while arginine levels remain low. Obviously, this pathological mechanism keeps supporting itself, resulting in severe glutamine muscle wasting.

In support of this view, several studies show that glutamine supplementation in cancer patients improves protein metabolism by decreasing protein breakdown and increasing protein synthesis rate in skeletal muscle (87). Several other studies show beneficial effects of arginine supplementation in catabolic states on protein turnover (88-90). Arginine supplementation was also effective in increasing fat free mass in advanced cancer patients with cachexia. Maintenance

of lean body mass should lead to improved survival and decreased morbidity in cancer patients (91). Arginine supplementation also increases glutamine levels in muscle and plasma. However, the observed physiological conversion of arginine to glutamine is to small to explain the large increase in glutamine production (92), suggesting that the corrected arginine levels do not require glutamine output anymore. Furthermore, glutamine enriched diets replete host glutamine stores and support muscle glutamine metabolism without stimulating tumor growth (93). Oral glutamine administration, may even decrease tumor growth by enhancing immune function, through support of host glutamine stores (94). Also, arginine supplementation has been showed to retard tumor growth by enhancing immune function. In a study in severe malnourished cancer patients perioperative arginine supplementation even decreased cancer recurrence and prolonged survival (95).

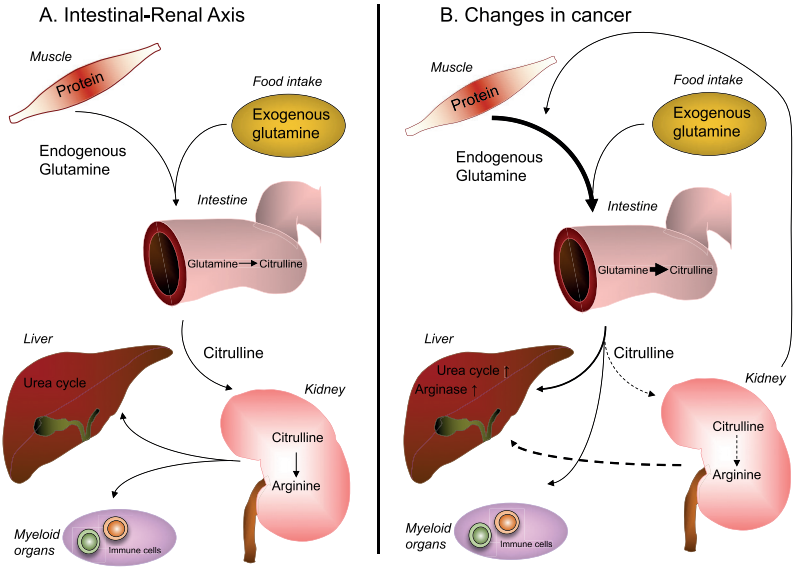

FIGURE 3 | The intestinal-renal axis in (**A**) physiological conditions and (**B**) in the presence of cancer. In figure B, muscle glutamine output and citrulline production increase, however arginine synthesis decreases and liver amino acid turnover rises.

However, arginine supplementation in patients with cancer show controversial results and administration of arginine to patients with advanced metastatic cancer may have negative oncologic effects (96,97). Therefore, arginine should

not be given to patients with progressive malignant disease. Glutamine, which is the main precursor for arginine *de novo* synthesis, is described to be a promising alternative and may be a save way to overcome the arginine deficiency in cancer patients (98). Though, arginine enriched nutrition administration in the perioperatieve phase may promote the immune system and may improve oncological outcome (95).

Role of arginine metabolism in other immune suppressive cachectic conditions

It is generally known that patients with other immune suppressive and inflammatory diseases may develop cachexia (99). We explained that malignant tumors have sophisticated mechanisms to escape immune response. Among these the induction of MDSC, which, through depletion of arginine by the enzymes arginase I and iNOS, inhibit specific signaling molecules and arrest the T-cell cycle, resulting in a stage of tolerance. This phenomenon however is not unique to cancer and is linked to other diseases associated with cachexia. MDSC are detected in patients with traumatic stress, chronic obstructive pulmonal disease (COPD), acute and chronic inflammation, sepsis, chronic infections, and several other conditions (100). In the same patients, arginase activity and iNOS activity are increased and arginine levels are decreased. Furthermore, proteolysis is increased in these patients and they show muscle wasting, while arginine levels remain low. This suggests that also in these patients arginine *de novo* synthesis is inadequate, secondary to decreased citrulline and glutamine supply (101-104). Subsequently, the immune function of T-cells in patients with these alterations in arginine/NO metabolism is impaired (105). MDSC are detected in other patient populations with immune dysfunction and severe cachexia as well, like AIDS patients (106). When these patients receive an arginine (and glutamine) enriched supplement, muscle mass acquisition and body weight increase, in addition T-cell counts improve (107). While the underlying mechanism is unclear, it is presumed that the presence of adequate arginine (and glutamine) levels enhance protein synthesis.

Testing the hypothesis

The proposed effect of an arginine deficiency on protein turnover in cancer patients, caused by MDSC, may be a plausible explanation for the host skeletal muscle glutamine depletion characteristic for cachexia. To test whether an arginine deficiency state underlie the development of cachexia, we propose the following experiments. According to our hypothesis, the arginine deficiency in cachectic cancer patients is caused by MDSC producing arginase and iNOS. The presence of these cells should be determined in tissues of patients with cancer cachexia. The population MDSC found in humans is very heterogeneous and examining the presence of these cells in all known potential tissue, like spleen, liver and lymphatic systems, is not compatible with ethical grounds. Human MDSC are CD11b+, CD33+ and HLA-DR-/low with granulocytic and monocytic subtypes, also accumulate in blood and the tumor environment, and these specimens may be analyzed more sufficient in humans. The presence of these cells is correlated to the stage of the malignant disease and tumor burden in various cancer types. As stated before, tumor burden is correlated to muscle glutamine content as well and thus might be correlated to cachectic muscle wasting. Subsequently MDSC's arginase and iNOS expression should be analyzed and the presence of the cells should be correlated to arginine concentrations in the cachectic patient. Furthermore, in the presence of these cells, decreased arginine levels should match with an increased glutamine output from skeletal muscle, which again should correlate with the state of cachexia. Ultimately, inhibiting the MDSC by new therapies or specialized nutrition, such as immune-enhancing nutrition containing arginine and the arginase inhibitor fish oil, might lead to a reduction in muscle wasting in cancer patients or even may lead to prevention of the cachectic process. Similarly, also in the other cachexia inducing diseases, such as COPD, chronic infections and AIDS, determination of the MDSC, arginine concentration and protein turnover may help to confirm the role of the arginine deficiency in the development of cachexia in these diseases.

Conclusion

The arginine deficiency – cancer cachexia hypothesis offers an explanation for the association between immunosuppressive states, caused by an arginine deficiency, and muscle wasting in cancer cachexia. A large component of the pathological changes in cancer cachexia can be ascribed to the arginine deficiency, glutamine releasing muscle wasting and excessive changes in the amino acid metabolism. The central role of the arginase and iNOS producing MDSC in our hypothesis is in accordance with other studies ascribing an important role to a compromised immune system in cachectic conditions. In other words, the accumulation of MDSC in cancer patients may affect immune function and protein turnover by depleting arginine levels and extract glutamine from skeletal muscle. We hope that our arginine deficiency – cancer cachexia hypothesis will generate pro-creative research that extend our understanding of the pathophysiology of cancer cachexia and may contribute to the design of preventive or therapeutic intervention strategies.

References

1. Evans WJ, Morley JE, Argiles J et al. Cachexia:a new definition. Clin. Nutr 2008;27(6):793-799.
2. Argiles JM, Busquets S, Moore-Carrasco R, Figueras M, Almendro V, Lopez-Soriano FJ. Targets in clinical oncology: the metabolic environment of the patient. Front Biosci 2007;12:3024-3051.
3. Van Cutsem E, Arends J. The causes and consequences of cancer-associated malnutrition. Eur J Oncol Nurs 2005;9 Suppl 2:S51-S63.
4. Talamonti MS, Kim SP, Yao KA et al. Surgical outcomes of patients with gastric carcinoma: the importance of primary tumor location and microvessel invasion. Surgery 2003;134(4):720-727.
5. Tisdale MJ. Cancer cachexia. Curr Opin Gastroenterol 2010;26(2):146-151.
6. Faber F. Immunological perspectives on nutritional support during cancer. PhD Thesis, Utrecht University: Utrecht, November 2011.
7. Fearon KC. The Sir David Cuthbertson Medal Lecture 1991. The mechanisms and treatment of weight loss in cancer. Proc Nutr Soc 1992;51(2):251-265.
8. Tisdale MJ. Mechanisms of cancer cachexia. Physiol Rev 2009;89(2):381-410.
9. Eley HL, Skipworth RJ, Deans DA, Fearon KC, Tisdale MJ. Increased expression of phosphorylated forms of RNA-dependent protein kinase and eukaryotic initiation factor 2alpha may signal skeletal muscle atrophy in weight-losing cancer patients. Br J Cancer 2008:98(2):443-449.
10. Jiang HY, Wek RC. GCN2 phosphorylation of eIF2alpha activates NFkappaB in response to UV irradiation. Biochem J 2005;385(Pt 2):371-380.
11. Mader S, Lee H, Pause A, Sonenberg N. The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4 gamma and the translational repressors 4E-binding proteins. Mol Cell Biol 1995;15(9):4990-4997.
12. Eley HL, Skipworth RJ, Deans DA, Fearon KC, Tisdale MJ. Effect of branched-chain amino acids on muscle atrophy in cancer cachexia. Br J Cancer 2007;98(2):443-449.
13. Khal J, Hine AV, Fearon KC, Dejong CH, Tisdale MJ. Increased expression of proteasome subunits in skeletal muscle of cancer patients with weight loss. Int J Biochem Cell Biol 2005;37(10):2196-2206.
14. Gomes MD, Lecker SH, Jagoe RT, Navon A, Goldberg AL. Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. Proc Natl Acad Sci USA 2001;98(25):14440-14445.
15. Bodine SC, Latres E, Baumhueter S et al. Identification of ubiquitin ligases required for skeletal muscle atrophy. Science 2001;294(5547):1704-1708.
16. Williams A, Sun X, Fischer JE, Hasselgren PO. The expression of genes in the ubiquitin-proteasome proteolytic pathway is increased in skeletal muscle from patients with cancer. Surgery 1999;126(4):744-749.
17. de Blaauw I, Heeneman S, Deutz NE, von Meyenveldt MF. Increased whole-body protein and glutamine turnover in advanced cancer is not matched by an increased muscle protein and glutamine turnover. J Surg Res1997;68(1):44-55.
18. Acharyya S. Cancer cachexia is regulated by selective targeting of skeletal muscle gene products. J Clin Invest 2004;114(3):370-378.

19. Morley JE, Thomas DR, Wilson MM. Cachexia: pathophysiology and clinical relevance. Am J Clin Nutr 2006;83(4):735-743.

20. Argiles JM, Busquets S, Lopez-Soriano FJ. Cytokines in the pathogenesis of cancer cachexia. Curr Opin Clin Nutr Metab Care 2003;6(4):401-406.

21. Schaap LA, Pluijm SM, Deeg DJ et al. Higher inflammatory marker levels in older persons: associations with 5-year change in muscle mass and muscle strength. J Gerontol A Biol Sci Med Sci 2009;64(11):1183-1189.

22. Lang CH, Frost RA, Nairn AC, MacLean DA, Vary TC. TNF-alpha impairs heart and skeletal muscle protein synthesis by altering translation initiation. Am J Physiol Endocrinol Metab 2002;282(2):E336-E347.

23. Buck M, Chojkier M. Muscle wasting and dedifferentiation induced by oxidative stress in a murine model of cachexia is prevented by inhibitors of nitric oxide synthesis and antioxidants. EMBO J 1996;15(8):1753-1765.

24. Li YP, Reid MB. NF-kappaB mediates the protein loss induced by TNFalpha in differentiated skeletal muscle myotubes. Am J Physiol Regul Integr Comp Physiol 2000;279(4):R1165-R1170.

25. Lang CH, Frost RA, Nairn AC, MacLean DA, Vary TC. TNF-alpha impairs heart and skeletal muscle protein synthesis by altering translation initiation. Am J Physiol Endocrinol Metab 2002;282(2):E336-E347.

26. Guttridge DC, Mayo MW, Madrid LV, Wang CY, Baldwin AS Jr. NF-kappaB-induced loss of MyoD messenger RNA: possible role in muscle decay and cachexia. Science 2000;289(5488):2363-2366.

27. Llovera M, Carbo N, Lopez-Soriano J et al. Different cytokines modulate ubiquitin gene expression in rat skeletal muscle. Cancer Lett 1998;133(1):83-87.

28. Argiles JM, Busquets S, Lopez-Soriano FJ. Cytokines in the pathogenesis of cancer cachexia. Curr Opin Clin Nutr Metab Care 2003;6(4):401-406.

29. Nieves C Jr, Langkamp-Henken B. Arginine and immunity: a unique perspective. Biomed Pharmacother 2002;56(10):471-482.

30. Rodriguez PC, Quiceno DG, Zabaleta J et al. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. Cancer Res 2004;64(16):5839-5849.

31. Taheri F, Ochoa JB, Faghiri Z et al. L-Arginine regulates the expression of the T-cell receptor zeta chain (CD3zeta) in Jurkat cells. Clin Cancer Res 2001;7(3 Suppl):958s-965s.

32. Peranzoni E, Marigo I, Dolcetti L et al. Role of arginine metabolism in immunity and immunopathology. Immunobiology 2007;212(9-10):795- 812.

33. Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. J Immunol 2009;182(8):4499-4506.

34. Rodriguez PC. T cell dysfunction in cancer: role of myeloid cells and tumor cells regulating amino acid availability and oxidative stress. Semin Cancer 2006;16(1):66-72.

35. Mazzoni A, Bronte V, Visintin A et al. Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. J Immunol 2002;168(2):689-695.

36. Morris SM Jr. Enzymes of arginine metabolism. J Nutr 2004;134(10 Suppl):2743S-2747S.

37. Zea AH, Rodriguez PC, Atkins MB et al. Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. Cancer Res 2005;65(8):3044-3048.

38. Deignan JL, Livesay JC, Yoo PK et al. Ornithine deficiency in the arginase double knockout mouse. Mol Genet Metab 2006;89(1-2):87-96.

39. Rodriguez PC, Ernstoff MS, Hernandez C et al. Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes. Cancer Res 2009;69(4):1553-1560.

40. Luiking YC, Engelen MP, Deutz NE. Regulation of nitric oxide production in health and disease. Curr Opin Clin Nutr Metab Care 2010;13(1):97-104.

41. Calcerrada P, Peluffo G, Radi R. Nitric Oxide-derived Oxidants with a Focus on Peroxynitrite: Molecular Targets, Cellular Responses and Therapeutic Implications. Curr Pharm Des 2011;17(35):3905-3932.

42. Wu G, Morris SM Jr. Arginine metabolism: nitric oxide and beyond. Biochem J 1998;336(Pt1):1-17.

43. Rennie MJ, MacLennan PA, Hundal HS et al. Skeletal muscle glutamine transport, intramuscular glutamine concentration, and muscle-protein turnover. Metabolism 1989;38(8Suppl1):47-51.

44. Cynober L, Moinard C, De Bandt JP. The 2009 ESPEN Sir David Cuthbertson. Citrulline: a new major signaling molecule or just another player in the pharmaconutrition game? Clin Nutr 2010;29(5):545-551.

45. Moinard C, Nicolis I, Neveux N, Darquy S, Benazeth S, Cynober L. Dose-ranging effects of citrulline administration on plasma amino acids and hormonal patterns in healthy subjects: the Citrudose pharmacokinetic study. Br J Nutr 2008;99(4):855-862.

46. Rodriguez PC, Ernstoff MS, Hernandez C et al. Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes. Cancer Res 2009;69(4):1553-1560.

47. Lee J, Ryu H, Ferrante RJ, Morris SM Jr, Ratan RR. Translational control of inducible nitric oxide synthase expression by arginine can explain the arginine paradox. Proc Natl Acad USA 2003;100(8):4843-4848.

48. Lu W, Laszlo CF, Miao Z, Chen H, Wu S. The role of nitric-oxide synthase in the regulation of UVB light-induced phosphorylation of the alpha subunit of eukaryotic initiation factor 2. J Biol Chem 2009;284(36):24281-24288.

49. Tong L, Heim RA, Wu S. Nitric oxide: a regulator of eukaryotic initiation factor 2 kinases. Free Radic Biol Med 2011;50(12):1717-1725.

50. Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. Nat Rev Immunol 2005;5(8):641-654.

51. Bauchart-Thevret C, Cui L, Wu G, Burrin DG. Arginine-induced stimulation of protein synthesis and survival in IPEC-J2 cells is mediated by mTOR but not nitric oxide. Am J Physiol Endocrinol Metab 2010;299(6):E899-E909.

52. Yao K, Yin YL, Chu W et al. Dietary arginine supplementation increases mTOR signaling activity in skeletal muscle of neonatal pigs. J Nutr 2008;138(5):867-872.

53. Frost RA, Nystrom GJ, Lang CH. Endotoxin and interferon-gamma inhibit translation in skeletal muscle cells by stimulating nitric oxide synthase activity. Shock 2009;32(4):416-426.

54. Kim YM, Son K, Hong SJ. Inhibition of protein synthesis by nitric oxide correlates with cytostatic activity: nitric oxide induces phosphorylation of initiation factor eIF-2 alpha. Mol Med 1998;4(3):179-190.

55. Di MS, Mazroui R, Dallaire P et al. NF-kappa B-mediated MyoD decay during muscle wasting requires nitric oxide synthase mRNA stabilization, HuR protein, and nitric oxide release. Mol Cell Biol 2005;25(15):6533-6545.

56. Matata BM, Galinanes M. Peroxynitrite is an essential component of cytokines production mechanism in human monocytes through modulation of nuclear factor-kappa B DNA binding activity. J Biol Chem 2002;277(3):2330-2335.

57. Frank JW, Escobar J, Nguyen HV et al. Oral N-carbamylglutamate supplementation increases protein synthesis in skeletal muscle of piglets. J Nutr 2007;137(2):315-319.

58. Herningtyas EH, Okimura Y, Handayaningsih AE et al. Branched-chain amino acids and arginine suppress MaFbx/atrogin-1 mRNA expression via mTOR pathway in C2C12 cell line. Biochem Biophys Acta 2008;1780(10):1115-1120.

59. Tan B, Yin Y, Liu Z et al. Dietary L-arginine supplementation increases muscle gain and reduces body fat mass in growing-finishing pigs. Amino Acids 2009;37(1):169-175.

60. Gomes-Marcondes MC, Tisdale MJ. Induction of protein catabolism and the ubiquitin-proteasome pathway by mild oxidative stress. Cancer Lett 2002;180(1):69-74.

61. Bar-Shai M, Carmeli E, Coleman R et al. The effect of hindlimb immobilization on acid phosphatase, metalloproteinases and nuclear factor-kappaB in muscles of young and old rats. Mech Ageing Dev 2005;126(2):289-297.

62. Bar-Shai M, Reznick AZ. Peroxynitrite induces an alternative NF-kappaB activation pathway in L8 rat myoblasts. Antioxid Redox Signal 2006;8(3-4):639-652.

63. Whitehouse AS, Tisdale MJ. Increased expression of the ubiquitinproteasome pathway in murine myotubes by proteolysis-inducing factor (PIF) is associated with activation of the transcription factor NF-kappaB. Br J Cancer 2003;89(6):1116-1122.

64. Breuillard C, Belabed L, Bonhomme S et al. Arginine availability modulates arginine metabolism and TNFalpha production in peritoneal macrophages from Zucker Diabetic Fatty rats. Clin. Nutr 2011, doi:10.1016/j.clnu.2011.11.012.

65. Fan XY, van den Berg A, Snoek M et al. Arginine deficiency augments inflammatory mediator production by airway epithelial cells . Respir Res 2009;10:62.

66. Hall DT, Ma JF, Marco SD, Gallouzi IE. Inducible nitric oxide synthase (iNOS) in muscle wasting syndrome, sarcopenia, and cachexia. Aging 2011;3(8):702-715.

67. Dodson S, Baracos VE, Jatoi A et al. Muscle wasting in cancer cachexia: clinical implications, diagnosis, and emerging treatment strategies. Annu Rev Med 2011;62:265-279.

68. Ramamoorthy S, Donohue M, Buck M. Decreased Jun-D and myogenin expression in muscle wasting of human cachexia. Am. J. Physiol Endocrinol. Metab 2009;297(2):E392-E401.

69. Visigalli R, Bussolati, O, Sala R et al. The stimulation of arginine transport by TNFalpha in human endothelial cells depends on NF-kappaB activation. Biochim Biophys Acta 2004;1664(1):45-52.

70. Li JM, Fan LM, Christie MR, Shah AM. Acute tumor necrosis factor alpha signaling via NADPH oxidase in microvascular endothelial cells: role of p47phox phosphorylation and binding to TRAF4. Mol Cell Biol 2005;25(6):2320-2330.

71. Guttridge DC, Mayo MW, Madrid LV, Wang CY, Baldwin AS Jr. NF-kappaB-induced loss of MyoD messenger RNA: possible role in muscle decay and cachexia. Science 2000;289(5488):2363-2366.

72. Hardin BJ, Campbell KS, Smith JD et al. TNF-alpha acts via TNFR1 and muscle-derived oxidants to depress myofibrillar force in murine skeletal muscle. J Appl Physiol 2008;104(3):694-699.

73. Chen DW, Wei FZ, Zhang YC, Ou JM, Xu J. Role of enteral immunonutrition in patients with gastric carcinoma undergoing major surgery. Asian J Surg 2005;28(2):121-124.

74. de Blaauw I, Heeneman S, Deutz NE, von Meyenfeldt MF. Increased wholebody protein and glutamine turnover in advanced cancer is not matched by an increased muscle protein and glutamine turnover. J Surg Res 1997;68(1):44-55.

75. Vissers YL, von Meyenfeldt MF, Luiking YC, Dejong CH, Deutz NE. Interorgan synthesis of arginine is down-regulated in tumor-bearing mice undergoing surgical trauma. Metabolism, 2008;57(7):896-902.

76. Noe JE. L-glutamine use in the treatment and prevention of mucositis and cachexia: a naturopathic perspective. Integr Cancer Ther 2009;8(4);409-415.

77. Chen MK, Espat NJ, Bland KI, Copeland EM III, Souba WW. Influence of progressive tumor growth on glutamine metabolism in skeletal muscle and kidney. Ann Surg 1993;217(6):655-666.

78. van de Poll MC, Siroen MP, van Leeuwen PA et al. Interorgan amino acid exchange in humans: consequences for arginine and citrulline metabolism. Am J Clin Nutr 2007;85(1):167-172.

79. Corbello P Sr, Darronqui E, Constantin J, da Silva MH, Yamamoto NS, Bracht A. The urea cycle and related pathways in the liver of Walker-256 tumor-bearing rats. Biochim Biophys Acta 2004;1688(3):187-196.

80. Espat NJ, Copeland EM, Souba WW. Accelerated hepatic arginine transport in the tumor-bearing rat. Ann Surg Oncol 1994;1(2):147-156.

81. Ilkovitch D, Lopez DM. The liver is a site for tumor-induced myeloidderived suppressor cell accumulation and immunosuppression. Cancer Res 2009;69(13):5514-5521.

82. Prins HA, Houdijk AP, Wiezer MJ et al. Reduced arginine plasma levels are the drive for arginine production by the kidney in the rat. Shock 1999;11(3):199-204.

83. Bansal V, Rodriguez P, Wu G et al. Citrulline can preserve proliferation and prevent the loss of CD3 zeta chain under conditions of low arginine. JPEN J Parenter Enteral Nutr 2004;28(6):423-430.

84. Amos JA, Fleming BC, Gusella JF, Jacoby LB. Relative argininosuccinate synthetase mRNA levels and gene copy number in canavanine-resistant lymphoblasts. Biochem Biophys Acta 1984;782(3):247-253.

85. Boyce FM III, Freytag SO. Regulation of human argininosuccinate synthetase gene: induction by positive-acting nuclear mechanism in canavanine-resistant cell variants. Somat Cell Mol Genet 1989;15(2):113-121.

86. Irr JD, Jacoby LB. Control of argininosuccinate synthetase by arginine in human lymphoblasts. Somatic. Cell Genet 1978;4(1):111-124.

87. Yoshida S, Kaibara A, Ishibashi N, Shirouzu K. Glutamine supplementation in cancer patients. Nutrition 2001;17(9):766-768.

88. Bruins MJ, Soeters PB, Lamers WH, Deutz NE. L-arginine supplementation in pigs decreases liver protein turnover and increases hindquarter protein turnover both during and after endotoxemia. Am J Clin Nutr 2002;75(6):1031-1044.

89. Cui XL, Iwasa M, Iwasa Y et al. Effects of dietary arginine supplementation on protein turnover and tissue protein synthesis in scald-burn rats. Nutrition 1999;15(7-8):563-569.

90. Hnia K, Gayraud J, Hugon G et al. L-arginine decreases inflammation and modulates the nuclear factor-kappaB/matrix metalloproteinase cascade in mdx muscle fibers. Am J Pathol 2008;172(6):1509-1519.

91. May PE, Barber A, D'Olimpio JT, Hourihane A, Abumrad NN. Reversal of cancer-related wasting using oral supplementation with a combination of beta-hydroxy-beta-methylbutyrate, arginine, and glutamine. Am J Surg 2002;183(4):471-479.

92. Yoshida S, Ishibashi N, Noake T, Shirouzu Y, Oka T, Shirouzu K. Glutamine and arginine metabolism in tumor-bearing rats receiving total parenteral nutrition. Metabolism 1997;46(4):370-3.

93. Klimberg VS, Souba WW, Salloum RM et al. Glutamine-enriched diets support muscle glutamine metabolism without stimulating tumor growth. J Surg Res 1990;48(4):319-323.

94. Fahr MJ, Kornbluth J, Blossom S, Schaeffer R, Klimberg VS. Harry M. Vars Research Award. Glutamine enhances immunoregulation of tumor growth. JPEN J Parenter Enteral Nutr 1994;18(6):471-476.

95. Buijs N, van Bokhorst-de van der Schueren MA, Langius JA et al. Perioperative arginine-supplemented nutrition in malnourished patients with head and neck cancer improves long-term survival. Am J Clin Nutr 2010;92(5):1151-1156.

96. Ascierto PA, Scala S, Castello G et al. Pegylated arginine deiminase treatment of patients with metastatic melanoma: results from phase I and II studies. J Clin Oncol 2005;23(30):7660-76668.

97. Glazer ES, Piccirillo M, Albino V et al. Phase II study of pegylated arginine deiminase for nonresectable and metastatic hepatocellular carcinoma. J Clin Oncol 2010;28(13):2220-2226.

98. Kuhn KS, Muscaritoli M, Wischmeyer P, Stehle P. Glutamine as indispensable nutrient in oncology: experimental and clinical evidence. Eur J Nutr 2010;49(4):197-210.

99. von Haehling S, Anker SD. Cachexia as a major underestimated and unmet medical need: facts and numbers. J Cachexia Sarcopenia Muscle 2010;1(1):1-5.

100. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol 2009;9(3):162-174.

101. Kao CC, Bandi V, Guntupalli KK, Wu M, Castillo L, Jahoor F. Arginine, citrulline and nitric oxide metabolism in sepsis. Clin Sci (Lond) 2009;117(1):23-30.

102. Maarsingh H, Pera T, Meurs H. Arginase and pulmonary diseases. Naunyn Schmiedebergs Arch Pharmacol 2008;378(2):171-184.

103. Ochoa JB, Bernard AC, O'Brien WE et al. Arginase I expression and activity in human mononuclear cells after injury. Ann Surg 2001;233(3):393-399.

104. Pribis JP, Zhu X, Vodovotz Y, Ochoa JB. Systemic arginine depletion after a murine model of surgery or trauma. JPEN J Parenter Enteral Nutr 2012;36(1):53-59.

105. Rodriguez PC, Zea AH, Culotta KS, Zabaleta J, Ochoa JB, Ochoa AC. Regulation of T cell receptor CD3zeta chain expression by L-arginine. J Biol Chem 2002;277(24):21123-21129.

106. Anthony DD, Umbleja T, Aberg JA et al. Lower peripheral blood CD14+ monocyte frequency and higher CD34+ progenitor cell frequency are associated with HBV vaccine induced response in HIV infected individuals. Vaccine 2011;29(19):3558-3563.

107. Clark RH, Feleke G, Din M et al. Nutritional treatment for acquired immunodeficiency virusassociated wasting using beta-hydroxy beta-methylbutyrate, glutamine, and arginine: a randomized, double-blind, placebo-controlled study. JPEN J Parenter Enteral Nutr 2000;24(3):133-139.

Adaptations of arginine's intestinal-renal axis in cachectic tumor-bearing rats

Buijs N
Vermeulen MAR
Weeda VB
Bading JR
Houdijk APJ
Van Leeuwen PAM

7

Published in:
Nutrition and Cancer 2015; 67(5): 713-720.

abstract

Malignancies induce disposal of arginine, an important substrate for the immune system. To sustain immune function, the tumor-bearing host accelerates arginine's intestinal-renal axis by glutamine mobilization from skeletal muscle and this may promote cachexia. Glutamine supplementation stimulates arginine production in healthy subjects. Arginine's intestinal-renal axis and the effect of glutamine supplementation in cancer cachexia have not been investigated. This study evaluated the longterm adaptations of the interorgan pathway for arginine production following the onset of cachexia and the metabolic effect of glutamine supplementation in the cachectic state. Fischer-344 rats were randomly divided into a tumor-bearing group (n=12), control group (n=7) and tumor-bearing group receiving a glutamine-enriched diet (n=9). Amino acid fluxes and net fractional extractions across intestine, kidneys, and liver were studied. Compared to controls, the portal-drained viscera of tumor-bearing rats took up significantly more glutamine and released significantly less citrulline. Renal metabolism was unchanged in the cachectic tumor-bearing rats compared with controls. Glutamine supplementation had no effects on intestinal and renal adaptations. In conclusion, in the cachectic state, an increase in intestinal glutamine uptake is not accompanied by an increase in renal arginine production. The adaptations found in the cachectic, tumor-bearing rat do not depend on glutamine availability.

Introduction

Cachexia is a complex catabolic state seen in up to eighty percent of patients with advanced cancer (1). It is associated with increased morbidity and mortality, and characterized by a loss and redistribution of the body's protein content resulting in muscle wasting. The etiology of this syndrome seems of multifactorial origin, but mechanisms remain unclear. This catabolic state is thought to occur in response to immune interactions between tumor and host (2).

Arginine and its derivate nitric oxide (NO) are important mediators of the anti-tumor immune response and metabolism (3). However, tumors develop a protection mechanism against the anti-tumor immune response by reducing arginine availability (4). The enzyme arginase is highly expressed during tumor development and arginase activity is found to be increased by 6-10 fold, resulting in an arginine waste (5,6).

Under physiologic circumstances endogenous arginine production results from *de novo* synthesis of arginine via the intestinal-renal axis. Glutamine is the precursor for this pathway and the most abundant amino acid in the body, with its main pool in skeletal muscle (7). Glutamine is converted into citrulline in the intestine and citrulline in its turn is used for arginine synthesis in the kidneys (8).

Previous studies in tumor-bearing rats have shown adaptations of arginine's intestinal-renal axis in different phases of malignant disease. In the first phase of malignant disease, arginine levels decrease without alterations in intestinal or renal metabolism. In a more advanced phase, the onset of cachexia is preceded by increases in glutamine release from muscle and citrulline production in the intestine, with a subsequent increase in renal arginine production (9,10). Although these adaptations normalize arginine levels, tumor-induced arginine waste may continue to drive glutamine mobilization (11-13).

Cancer cachexia is characterized by increased glutamine release from muscle and low glutamine plasma concentrations (14). It has been suggested that, when arginine demand is high in the presence of a malignancy, glutamine is excessively released from skeletal muscle to form extra substrate for the intestinal-renal axis. When this becomes a chronic process, it results in muscle wasting and subsequently in cachexia (15,16).

Other studies have shown that in an environment with altered arginine metabolism, citrulline and glutamine may take over arginine's immune enhancing

capacity (17-19). Thus, the body is able to adapt to changes in substrate availability and to recruit other sources to maintain immune function and homeostasis. In the context of the current study, we hypothesized that the body does not sustain wasteful arginine synthesis by increasing glutamine and citrulline supply long-term, but instead readjust the intestinal-renal axis. Instead of using muscle-derived glutamine for redundant *de novo* arginine synthesis, the host may find alternative ways to support immune function and protein metabolism.

Glutamine supplementation in healthy subjects stimulates *de novo* arginine synthesis via the intestinal-renal axis (20). Although some studies suggest that glutamine supplementation may restore glutamine sufficiency in cachexia, we speculate that the adaptation of the intestinal-renal axis is not reversible by glutamine supplementation, since the initiating factor, arginine waste, is still present.

In this study, the primary aim was to investigate the long-term adaptation of the intestinal-renal axis in the tumor-bearing host. The secondary aim was to determine if extra glutamine could reverse these adaptations.

Materials & Methods

This is a preliminary study to explore the metabolism of glutamine, citrulline and arginine in the cachectic tumor-bearing host. The animal protocol in this study was approved by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee, where the experiments were conducted.

Animals

Immature, male, Fischer-344 rats (Charles River Breeding Laboratories, Wilmington, MA) with an initial weight of 200-210 grams were used in this study. They were housed in a temperature-controlled environment (21 $\pm$ 2^{0}C) and subjected to a standard 12-hour light-dark cycle. After an acclimatization period of five days, rats were randomly divided into a tumor-bearing group (TBR; n=12) and a control group (n=7). To investigate whether the intestinal-renal axis in the tumor-bearing rat can be influenced by glutamine supplementation, we performed an additional experiment in tumor-bearing rats (TBR+GLN; n=9) receiving a glutamine-enriched enteral diet.

Tumor model

Sarcoma induced with methylcholanthrene (MCA) and serially passaged in our laboratory, was the tumor model chosen for this study. The MCA tumor is known to induce a cachectic state in the host and this experimental model has been widely used to study metabolic mechanisms in mice and rats. Implantation was performed by anesthetizing animals and inserting a 1 mg piece of tumor aseptically and subcutaneously in the caudal dorsal area of the TBR and TBR+GLN. Control rats received a sham operation.

During the acclimatization period, all rats received pulverized standard rat chow (Purina Mills, St Louis, MO, USA). Following implantation, the rats were housed individually in suspended wire cages designed to permit accounting of scattered food. Food and water intake were measured daily. The animals in the TBR group were matched to the animals of the TBR+GLN group and the control group to allow pair feeding. TBR rats were provided with ad libitum excess to food. The pair-fed animals were placed on a protocol that provided the amount as consumed by the TBR on the corresponding experimental day. TBR+GLN received the matched amount of water and food, but enriched with glutamine. The glutamine-enriched diet was prepared in our laboratory by adding 12.5% L-glutamine powder to the pulverized standard rat chow. The control group and the TBR group received the control diet, in which glutamine was replaced by asparagine (3.3%), serine (2.4%), glycine (3.9%), proline (2.6%), alanine (2.0%) to balance the nitrogen and caloric content of the glutamine-enriched diet.

Twenty-six days after implantation and 4-5 hours after removal of food, the rats underwent blood sampling. The animals were then sacrificed by intravenous overdose of pentobarbital and dissection was performed. Weights of body, tumor, and organs were measured. Tumor dimensions were measured externally with a skinfold caliper, and tumor weight was computed assuming an ellipsoidal shape. Tumor weight was calculated by taking ninety percent of the tumor volume and add 1.84, as previously validated (21).

Blood sampling and analysis

A laparotomy was performed on each rat following anesthetization with pentobarbital sodium (50 mg · kg^{-1} i.p.). Blood (1 ml per sample) was taken from the aorta and the hepatic, portal and renal veins using a technique previously described (22). Plasma amino acid concentrations were then determined by HPLC as previously described (23).

Blood flow measurements

Organ plasma flows were determined by a radiolabeled microsphere technique. The surgical and physical techniques by which the microsphere measurements were performed have been previously described in detail (24). In brief, 14 µm radiolabeled microspheres (^{85}Sr, 3M Health Care, St. Paul, MN, USA) were injected into the aorta, and reference blood samples were drawn from a femoral artery catheter. Adequate mixing of the microspheres in aortic blood was confirmed via bilateral comparison of radioactivity per gram of tissue in the kidneys. High statistical precision of the microsphere distributions was verified by estimating the number of microspheres in each tissue and blood sample. Arterial whole blood flow values were computed according to the "reference organ" technique (24). Mean organ blood flow was used for metabolic quantification, as recommended in the literature (25). Arterial plasma flow was computed by using hematocrit (Table 1).

TABLE 1 | Equations

Variable	Equation
Blood flow (F_b)	$F_b = F_a (C_o / C_a)$
Plasma flow (F_p)	$F_p = F_b$(1-hematocrit)
Portal-drained viscera flux (Q_{PDV})	$Q_{PDV} = ([A] - [PV]) * F_p(PV)$
Renal flux (Q_{REN})	$Q_{REN} = ([A] - [RV]) * (F_p(RV) * 2)$
Splanchnic flux ($Q_{SPLANCH}$)	$Q_{SPLANCH} = ([A] - [HV]) * F_p(HA + PV)$
Hepatic flux (Q_{HEP})	$Q_{HEP} = (Q_{PDV}) - (Q_{SPLANCH})$
Net FE portal-drained viscera (FE_{PDV})	$FE_{PDV} = ([A] - [PV]) / [A]$
Net FE kidneys (FE_{REN})	$FE_{REN} = ([A] - [RV]) / [A]$
Substrate Load Liver ($Load_{HEP}$)	$Load_{HEP} = (F_p(HA) * [A]) + (F_p(PV) * [PV])$
Net FE liver (FE_{HEP})	$FE_{HEP} = (Load - (F_p(HA) + F_p(PV)) * [HV]) / Load$

F_b, blood flow. F_a, reference flow (computed from reference blood weight and duration of withdrawal, assuming whole blood density of 1.069 g·mL^{-1}). C_o, organ count rate. C_a, reference blood count rate. F_p, plasma flow. Q, flux in nmol·min^{-1}. PDV, portal drained viscera. REN, renal. SPLANCH, splanchnic. HEP, hepatic. A, arterial. PV, portal vein. RV, renal vein. HV, hepatic vein. FE, fractional extraction in percent. Positive values resemble net uptake, negative values resemble net release.

Calculations

All equations used for calculating organ metabolism of the amino acids of interest are described in table 1. Amino acid fluxes across the portal-drained viscera (PDV) (representing mainly intestinal metabolism), liver and kidneys were calculated. Since changes in net amino acid flux may result solely from changes in organ flow, net fractional extraction (FE) was determined. Net protein breakdown in the PDV was estimated from the respective phenylalanine flux. Net protein breakdown in liver and kidney were equated with the respective valine fluxes, as described previously (13,26).

Statistical analysis

All statistical analyses were performed using SPSS 20.0 software (SPSS Inc., Chicago, IL). Data are presented as mean ± standard error of the mean (SEM). To test the significance of differences between groups, Student's *t*-test (in case of equal variances) or Welch test (in case of unequal variances) were used. Non-paired Student's *t*-test was used to determine if net FE rates differed significantly from zero. P-values < 0.05 were considered to be significant.

Results

Food intake, body weight, tumor weight and arterial amino acid concentrations

Food intake was constant at 16 ± 0.2 g $\cdot$ d^{-1} during the first 24 days and then declined slightly on the 25th day. The control group had a constant growth rate throughout the study. Total body weight (host + tumor) of the TBR exceeded that of the controls after day 16 (Figure 1A). At sacrifice, mean tumor weight was 53 ± 9 g or 17% of body weight. Non-tumor weight (total – tumor) was significantly lower in TBR than controls, implying that the TBR were cachectic by day 26 (Table 2; Figure 1A). Tumor growth and non-tumor weights did not differ significantly between the TBR and TBR+GLN groups (Figure 1B). Arterial glutamine concentrations in the TBR group were 19% lower than in the control group. There were no significant differences in citrulline and arginine plasma concentrations between the TBR and control groups. Although mean arterial glutamine concentration in the TBR+GLN group was even higher than in the control group, citrulline and arginine levels did not change significantly (Table 2).

TABLE 2 | Subject characteristics.

Group	CONTROL	TBR	TBR+GLN
Total Body weight (g)	291 ± 3	317 ± 7*	315 ± 2*
Tumor	-	53 ± 9	53 ± 2
Non-tumor weight (g)	291 ± 3	264 ± 11*	262 ± 9*
Organ weights (g)			
Portal drained viscera	13.41 ± 0.20	12.17 ± 0.54	11.89 ± 0.30
Small intestine	6.79 ± 0.20	6.93 ± 0.40	7.13 ± 0.31
Spleen	0.76 ± 0.03	1.32 ± 0.16*	1.36 ± 0.10*
Liver	9.70 ± 0.21	11.55 ± 0.22*	11.7 ± 0.20*
Kidney	2.04 ± 0.05	2.09 ± 0.06	2.07 ± 0.02
Arterial plasma concentrations ($nmol \cdot ml^{-1}$)			
Glutamine	698 ± 30	565 ± 21*	756 ± 33*
Citrulline	42 ± 3	44 ± 5	44 ± 4
Arginine	85 ± 7	82 ± 4	86 ± 3
Plasma flows ($ml \cdot min^{-1}$)			
Portal drained viscera	5.2 ± 0.6	13.6 ± 1.6*	13.5 ± 1.2*
Kidneys	5.0 ± 0.9	10.2 ± 1.6*	10.2 ± 1.2*
Liver	6.5 ± 0.6	17.4 ± 1.7*	14.7 ± 1.2*

Values are mean ± SEM on day 26 after tumor implantation; TBR tumor-bearing group; TBR+GLN tumor-bearing group with glutamine-enriched diet. Plasma flows were measured in separate identical groups (n=7 per group) * $P<0.05$ compared to the control group.

Organ plasma flow

Portal, hepatic and renal plasma flows increased in TBR compared with controls (Table 2). The change in hepatic blood flow in the tumor-bearing state resulted from an increase in both portal venous and hepatic arterial flows. Organ flows in the TBR+GLN group were comparable to those in the TBR group.

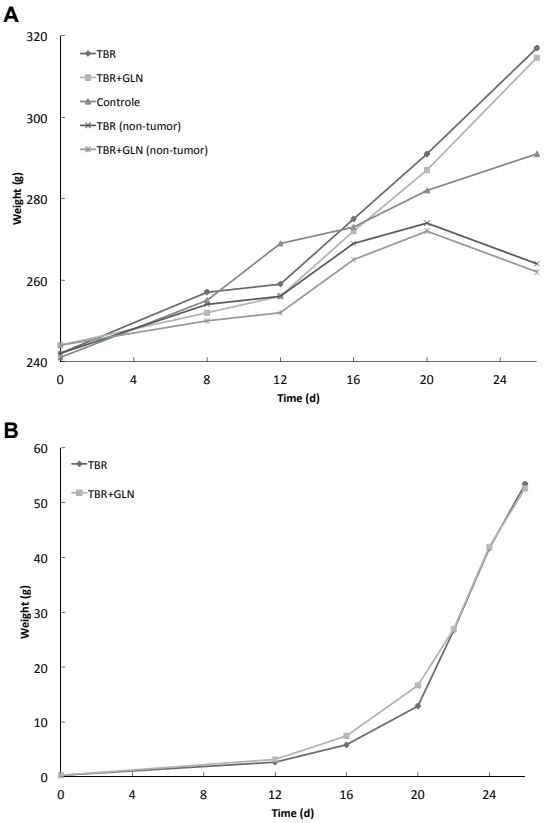

FIGURE 1 | (**A**) Mean body weight (g) of the rats in the control group, TBR group and TBR+GLN group with and without tumor weight. (**B**) Mean tumor weight (g) of rats in the TBR and TBR+GLN group.

Adaptations in the portal-drained viscera

The weight of the small intestine showed no significant differences among groups, while the spleen was enlarged in the TBR relative to the control group (Table 2). Despite lower glutamine plasma levels, the glutamine load to the PDV was higher in the TBR group as a result of the higher PDV blood flow. Glutamine utilization by the PDV was significantly increased in the presence of tumor, while citrulline release was significantly decreased (Figure 2). Glutamine FE by the PDV was decreased in the tumor-bearing rats (Table 3). Citrulline FE showed

a marked increase in TBR (-14% compared with -121% in controls), indicating a sharp decline in citrulline output. Net protein breakdown in the PDV in the tumor-bearing group was unchanged compared to the control group, i.e. phenylalanine flux was unchanged (Table 3).

TABLE 3 | Organ amino acid fluxes and net fractional extraction rates.

Group	CONTROL		TBR		TBR+GLN	
	Flux ($nmol \cdot min^{-1}$)	Net FE (%)	Flux ($nmol \cdot min^{-1}$)	Net FE (%)	Flux ($nmol \cdot min^{-1}$)	Net FE (%)
Portal drained viscera						
Glutamine	1366 ±158	37 ± 3	2031± 242*	26 ± 2*	2040 ± 426*	20 ± 4*
Citrulline	-253 ± 21	-121 ± 14	-35 ± 82*	-14 ±11*†	-195 ± 42	-35 ± 10
Phenylalanine	*-2 ± 18*		*-7 ± 46*		*-39 ± 84*	
Glutamate	*-129 ± 26*		*-379 ± 137*		*-255 ± 117*	
Alanine	*-1082 ± 115*		*-1318 ± 660*		*-2720 ± 777*	
Kidneys						
Citrulline	99 ± 19	45 ± 7	220 ± 72	35 ± 13	163 ± 46	34 ± 9
Arginine	-126 ± 20	-31 ± 5	-154 ± 43	-19 ± 5	-184 ± 68	-27 ± 10
Valine	*56 ± 28*		*79 ± 21*		*182 ± 44**	
Liver						
Glutamine	-438 ± 100	-14 ± 4	-171 ± 414	- 3± 6†	2230 ± 322*	19 ± 2*
Citrulline	134 ± 48	25 ± 9	-16 ± 67	-5 ± 11†	82 ± 54	10 ± 7†
Arginine	223 ± 45	34 ± 5	276 ± 76	7 ± 11†	473 ± 36*	34 ± 3
Valine	*34 ± 24*		*324 ± 87**		*324 ± 128**	

Values are mean ± SEM on day 26 after tumor implantation; TBR tumor-bearing group; TBR+GLN tumor-bearing group with glutamine-enriched diet. Positive values resemble net uptake, negative values resemble net release. * P<0.05 compared to the control group. † Not significantly different from zero.

The high glutamine uptake was accompanied by a trend towards an increase in glutamate release by the PDV in the TBR group. Alanine flux showed a non-significant increase in the TBR group compared to the control group (Table 3).

The glutamine-enriched diet had no effect on the intestinal-renal axis of tumor-bearing rats compared to control rats (Figure 2). Even with high glutamine plasma levels, the PDV did not release more citrulline in the TBR+GLN group compared to controls. Glutamate and alanine release from the PDV were higher in the TBR+GLN group compared to the control group, however this was not a significant increase (Table 3).

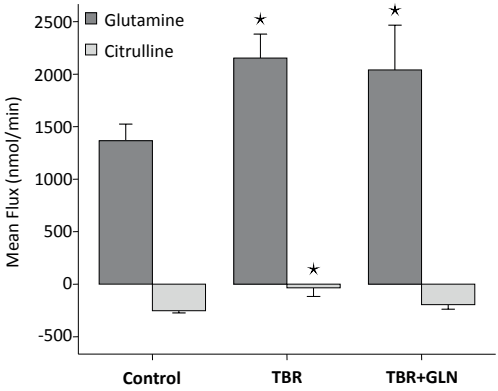

FIGURE 2 | Glutamine and citrulline flux across the portal-drained viscera (PDV). Values are mean ± SEM on day 26 after tumor implantation; Positive values resemble net uptake, negative values resemble net release. * P<0.05 compared to the control group.

Renal adaptations

Kidney weight did not differ between the control group and the tumor-bearing groups (Table 2). Citrulline and arginine kidney fluxes did not differ significantly among groups, although there appeared to be modest increases in citrulline uptake and arginine release in tumor bearers (Figure 3). However, citrulline and arginine FEs both trended toward decreased conversion (Table 3). Net renal protein breakdown did not differ between the TBR and control groups. The TBR+GLN group showed the same renal metabolism as the TBR group. However, net renal protein breakdown was reduced in the TBR+GLN group, as indicated by increased valine uptake (Table 3).

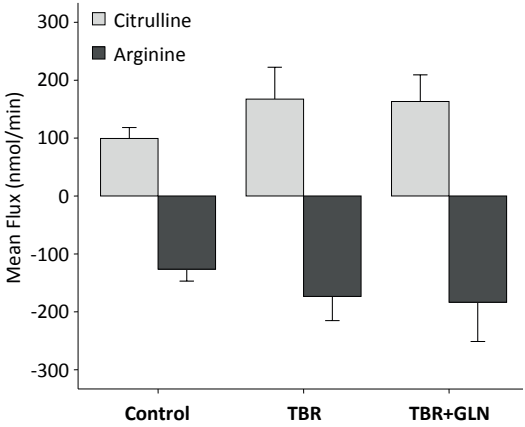

FIGURE 3 | Arginine and citrulline flux across the kidneys. Values are mean ± SEM on day 26 after tumor implantation; Positive values resemble net uptake, negative values resemble net release. * P<0.05 compared to the control group.

Hepatic adaptations

Liver weight was significantly increased in the TBR group compared to the control group. There were no significant differences in net amino acid fluxes across the liver between the TBR and control groups (Figure 4). Hepatic amino acid FEs showed small amounts of glutamine release and citrulline and arginine uptake in the control group. In the TBR group, glutamine, citrulline and arginine fluxes were not significantly different from zero. Net hepatic protein breakdown was reduced in the tumor-bearing rats, as indicated by an increase in valine uptake.

In the TBR+GLN group, liver weight showed a similar increase as the TBRs compared to the control group. However, in the TBR+GLN group, liver glutamine flux switched to strongly positive utilization compared to modest release by controls and near balance for TBRs (Figure 4). Hepatic glutamine FE for the TBR+GLN group showed high glutamine uptake relative to the other groups. Hepatic citrulline FE was also not significantly different from zero. Arginine FE was unchanged compared to the control group. Net hepatic protein breakdown was also reduced in the TBR+GLN group (Table 3).

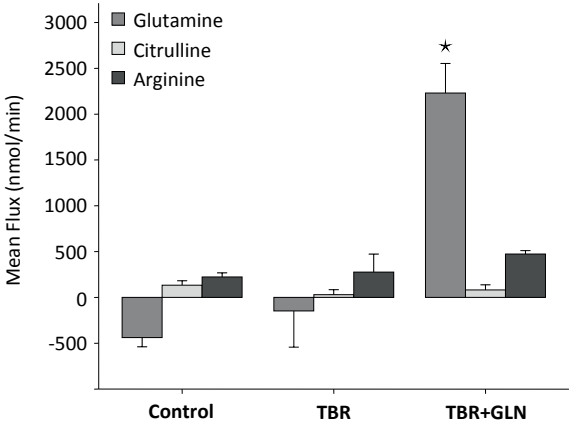

FIGURE 4 | Glutamine, citrulline and arginine flux across the liver. Values are mean ± SEM on day 26 after tumor implantation; Positive values resemble net uptake, negative values resemble net release. * $P<0.05$ compared to the control group.

Discussion

This experimental study shows that the intestinal-renal axis is adapted in cachectic tumor-bearing rats and extra glutamine does not reverse the adaptations. Unlike in pre-cachectic rats with MCA sarcomas, the intestine does not produce significantly more citrulline and kidneys do not release significantly more arginine in cachectic tumor-bearing rats than in controls (Figure 2). The administration of a glutamine-enriched diet did not alter tumor growth or weight loss and did not increase intestinal citrulline or renal arginine release compared to controls.

Cachexia is a highly negative prognostic factor in cancer and one third of all cancer patients die from this catabolic state rather than from the malignancy itself (27). Cachexia results from alterations in anti-tumor immune response and inflammation caused by tumor and host-derived factors.

As frequently described, arginine plays an important role in anti-tumor immune defense, since it is the primary substrate for cells of the immune system, and the precursor for NO, which regulates cell proliferation and immune responses (28). However, malignant tumors protect themselves by interfering with arginine metabolism. Malignant tumors recruit immature myeloid-derived suppressor cells (MDSC) that accumulate in the tumor environment, peripheral blood, liver

and organs of the immune system. These MDSC activate arginine catabolizing enzymes, which increases arginine demand (29). Eventually, the body has to adapt to this tumor-induced arginine waste in order to sustain its immune function (30).

In physiologic conditions, arginine is produced by the intestinal-renal axis. Glutamine is converted into citrulline in the intestine and citrulline is used as a substrate for *de novo* arginine synthesis in the kidneys. The intestinal-renal axis adapts to the ongoing arginine waste in the tumor-bearing state, depending on the phase of tumor development.

In the early phase of tumor growth, plasma arginine levels are low, while glutamine and citrulline levels remain normal. Ornithine levels are high in this phase of tumor growth, consistent with increased arginine catabolism (12). Intestinal glutamine utilization and citrulline efflux do not adapt in this early phase and *de novo* arginine production is unchanged (9,12). In a more advanced phase of tumor development, but still prior to signs of cachexia, muscle glutamine output and glutamine conversion to citrulline in the intestine increase (9,10,13). Consequently, arginine production increases and arginine plasma levels normalize (13). Ornithine levels remain high in this pre-cachectic phase, indicating that arginine catabolism is still high (12). In this advanced phase, muscle glutamine release is initiated to provide substrate for the intestinal-renal axis, which implies that the body adapts to the high demand for arginine in the tumor-bearing state. As this process proceeds, the glutamine pool in the muscles diminishes, contributing to muscle wasting and cancer cachexia.

Our study characterizes the intestinal-renal axis in the advanced tumor-bearing phase, when signs of cachexia are present. Our observations suggest that the body does not sustain the adaptations from the previous phase and finds alternative ways to overcome arginine waste. This occurs by an adaptation of intestinal amino acid metabolism, leading to decreased citrulline efflux. Renal arginine production from citrulline stays in balance, i.e. there is no long-term renal adaptation in the tumor-bearing state.

We found significantly increased glutamine utilization and significantly decreased citrulline release by the PDV. Similar to our results, other investigators have shown increased glutamine utilization in the tumor-bearing state (9,13,31). It is implied that glutamine turnover is increased in visceral organs and cells of the immune system (14,32). A major part of the immune system, known as the gut-associated lymphoid tissue (GALT) is located in the PDV, however in the presence of a

malignancy the immune function of the GALT is impaired (33-35). Arginine has been shown to be an important substrate for the GALT with stimulating effects on its immune function (36). As described above, MDSC accumulate in lymphoid tissues like the GALT, and deplete arginine availability in the tumor-bearing state (29). Consequently, the substrate supply for the GALT is lacking. Studies showed that glutamine and citrulline may function as substrates for the GALT and thus improve intestinal immunity when arginine is scarce (17,36). This may explain the observed increased glutamine utilization and significantly decreased citrulline release by the PDV in the cachectic tumor-bearing state. The trend towards a higher glutamate release from the PDV may support this, since glutamate is the end product of glutamine utilization by the immune cells of rats (37). Thus, the PDV would increase glutamine uptake and decreases citrulline release to generate substrate for the GALT.

In the non-cancerous state, dietary glutamine increases citrulline production in the intestine and subsequently increases renal *de novo* arginine synthesis (20). Thus, theoretically it should be possible to reverse the adaptation process of the intestinal-renal axis in cachectic tumor-bearing rats with a glutamine-enriched diet. However, the adaptations of the intestinal-renal axis in our cachectic tumor-bearing rats were not significantly altered by the administration of extra glutamine. The present study shows that the intestinal metabolic adaptation in tumor-bearing rats fed with a glutamine-enriched diet is similar to the adapted intestinal metabolism of tumor-bearing rats without a glutamine-enriched diet. Furthermore, the glutamine-enriched diet also did not affect renal citrulline-arginine metabolism.

A major effect of the glutamine-enriched diet was seen in the liver, which adapted to the increased glutamine availability by utilizing more glutamine. In the liver, glutamine is used for acid base regulation and hepatic ammonium detoxification, both very important processes during cancer cachexia. Hepatic glutamine flux was approximately zero in the TBR group and we assume that increased intestinal glutamine uptake in the tumor-bearing state depletes hepatic glutamine. Decreased glutamine concentrations in the liver of tumor-bearing rats are described in the literature (38). However, when administered enterally, glutamine is directly available for liver metabolism via the portal circulation and the 'first pass' eliminates 40-90% of the enterally administered glutamine (39). This may explain the high hepatic glutamine uptake. Furthermore, this may deplete glutamine supply from the diet to the muscles. Accordingly, weight

loss was similar in the TBR and TBR+GLN groups. Although it is unclear whether supplemental glutamine restores muscle glutamine during muscle wasting, findings from Boutry et al. also suggest that glutamine supplementation is not effective in counteracting glutamine release from muscle or muscle protein breakdown in endotoxemia (40).

Taken together, our results imply that metabolic alterations found in tumor-bearing rats with advanced cancer cachexia do not depend on glutamine availability, but rather seem to be an adaptation process to tumor-induced changes in the host. Future studies are necessary to further determine the complicated interorgan route of glutamine, citrulline and arginine in cancer cachexia, using in-depth research techniques to define the specific precursor functions in this pathway. Furthermore, it would be of great interest to explore the role of the immune system in the metabolic adaptations in the tumor-bearing state. Distinct insight in the adaptations of amino acid metabolism and the effect of supplemental components on metabolic pathways in the tumor-bearing host with cachexia offers opportunities to counteract nutritional and immunological deficiencies.

References

1. von Haehling S, Anker SD. Cachexia as a major underestimated and unmet medical need: facts and numbers. J Cachexia Sarcopenia Muscle 2010;1:1-5.
2. Burckart K, Beca S, Urban RJ, Sheffield-Moore M. Pathogenesis of muscle wasting in cancer cachexia: targeted anabolic and anticatabolic therapies. Curr Opin Clin Nutr Metab Care 2010;13:410-416.
3. Wu G, Morris SM Jr. Arginine metabolism: nitric oxide and beyond. Biochem J 1998;336:1-17.
4. Peranzoni E, Marigo I, Dolcettie L et al. Role of arginine metabolism in immunity and immunopathology. Immunobiology 200;212:795-812.
5. Deignan JL, Livesay JC, Yoo PK et al. Ornithine deficiency in the arginase double knockout mouse. Mol Genet Metab 2006;89:87-96.
6. Zea AH, Rodriguez PC, Atkins MB et al. Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. Cancer Res 2005;65:3044-3048.
7. Vermeulen MA, van de Poll MC, Ligthart-Melis GC, Dejong CH, van den Tol MP et al. Specific amino acids in the critically ill patient - exogenous glutamine/arginine: a common denominator? Crit Care MeD 2007;35:S568-S576.
8. van de Poll MC, Ligthart-Melis GC, Boelens PG et al. Intestinal and hepatic metabolism of glutamine and citrulline in humans. J Physiol 2007;58:819-827.
9. de Blaauw I, Deutz NE, van der Hulst RR, von Meyenfeldt MF. Glutamine depletion and increased gut permeability in nonanorectic, non-weight-losing tumor-bearing rats. Gastroenterology 1997;112:118-126.
10. Souba WW, Strebel FR, Bull JM et al. Interorgan glutamine metabolism in the tumor-bearing rat. J Surg Res 1988;44:720-6.
11. Hartman WJ, Prior RL. Dietary arginine deficiency alters flux of glutamine and urea cycle intermediates across the portal-drained viscera and liver of rats. J Nutr 1992;122:1472-1482.
12. Vissers YL, von Meyenfeldt MF, Luiking YC et al. Presence of tumour inhibits the normal post-operative response in arginine and NO production in non-cachectic mice. Clin Sci (Lond) 2007;112:527-532.
13. Vissers YL, von Meyenfeldt MF, Luiking YC, Dejong CH, Deutz NE. Interorgan synthesis of arginine is down-regulated in tumor-bearing mice undergoing surgical trauma. Metabolism 2008;57:896-902.
14. de Blaauw I, Heeneman S, Deutz NE, von Meyenfeldt MF. Increased whole-body protein and glutamine turnover in advanced cancer is not matched by an increased muscle protein and glutamine turnover. J Surg Res 1997;68:44-55.
15. Buijs N, Luttikhold J, Houdijk AP, van Leeuwen PA. The role of a disturbed arginine/NO metabolism in the onset of cancer cachexia: a working hypothesis. Curr Med Chem 2012;19:5278-5286.
16. Noe JE. L-glutamine use in the treatment and prevention of mucositis and cachexia: a naturopathic perspective. Integr Cancer Ther 2009;8:409-415.
17. Bansal V, Rodriguez P, Wu G et al.: Citrulline can preserve proliferation and prevent the loss of CD3 zeta chain under conditions of low arginine. JPEN J Parenter Enteral Nutr2004;28:423-430.
18. Wijnands KA, Vink H, Briede JJ et al. Citrulline a more suitable substrate than arginine to restore NO production and the microcirculation during endotoxemia. PLoS One 2012;7:e37439.
19. Wu GY, Brosnan JT. Macrophages can convert citrulline into arginine. Biochem J 1992;281(Pt 1):45-48.

20. Houdijk AP, van Leeuwen PA, Teerlink T et al. Glutamine-enriched enteral diet increases renal arginine production. JPEN J Parenter Enteral Nutr 1994;18:422-426.
21. Younes RN, Rogatko A, Vydelingum NA, Brennan MF. Effects of hypovolemia and transfusion on tumor growth in MCA-tumor-bearing rats. Surgery 1991;109:307-312.
22. Welbourne TC, Phromphetcharat V, Givens G, Joshi S. Regulation of interorganal glutamine flow in metabolic acidosis. Am J Physiol 1986;250:E457-E463.
23. Teerlink T, Van Leeuwen PA, Houdijk A. Plasma amino acids determined by liquid chromatography within 17 minutes. Clin Chem 1994;40:245-249.
24. Boelens PG, Houdijk AP, de Thouars HN et al. Plasma taurine concentrations increase after enteral glutamine supplementation in trauma patients and stressed rats. Am J Clin Nutr 2003;77:250-256.
25. Vermeulen MA, Ligthart-Melis GC, Buijsman R et al. Accurate perioperative flow measurement of the portal vein and hepatic and renal artery: a role for preoperative MRI? Eur J Radiol 2012;81:2042-2048.
26. Hallemeesch MM, Soeters PB, Deutz NE. Tracer methodology in whole body and organ balance metabolic studies: plasma sampling is required. A study in post-absorptive rats using isotopically labeled arginine, phenylalanine, valine and leucine. Clin Nutr 2000;19:157-163.
27. Van CE, Arends J. The causes and consequences of cancer-associated malnutrition. Eur J Oncol Nurs 2005; 9 Suppl 2:S51-S63.
28. Popovic PJ, Zeh HJ 3rd, Ochoa JB: Arginine and immunity. J Nutr 2007;137:1681S-1686S.
29. Raber P, Ochoa AC, Rodriguez PC. Metabolism of L-arginine by myeloid-derived suppressor cells in cancer: mechanisms of T cell suppression and therapeutic perspectives. Immunol Invest 2012;41:614-634.
30. Bansal V, Ochoa JB. Arginine availability, arginase, and the immune response. Curr Opin Clin Nutr Metab Care 2003;6:223-228.
31. Souba WW. Glutamine and cancer. Ann Surg 1993;218:715-728.
32. Chen MK, Espat NJ, Bland KI, Copeland EM 3rd, Souba WW. Influence of progressive tumor growth on glutamine metabolism in skeletal muscle and kidney. Ann Surg 1993;217:655-667.
33. Mowat AM, Millington OR, Chirdo FG. Anatomical and cellular basis of immunity and tolerance in the intestine. J Pediatr Gastroenterol Nutr 2004;39 Suppl 3:S723-S724.
34. Rabinovich GA, Gabrilovich D, Sotomayor EM. Immunosuppressive strategies that are mediated by tumor cells. Annu Rev Immunol 2007;25:267-296.
35. Yaguchi T, Sumimoto H, Kudo-Saito C et al. The mechanisms of cancer immunoescape and development of overcoming strategies. Int J Hematol 2011;93:294-300.
36. Fan J, Meng Q, Guo G, Xie Y, Xiu Y et al. Effects of enteral nutrition supplemented with glutamine on intestinal mucosal immunity in burned mice. Nutrition 2009;25:233-239.
37. Fernandes LC, Marques-da-Costa MM, Curi R. Metabolism of glucose, glutamine and pyruvate in lymphocytes from Walker 256 tumor-bearing rats. Braz J Med Biol Res 1994;27:2539-2543.
38. Hulsewe KW, Deutz NE, de Blaauw I et al. Liver protein and glutamine metabolism during cachexia. Proc Nutr Soc 1997;56:801-806.
39. Wernerman J. Glutamine supplementation. Ann Intensive Care 2011;1:25.
40. Boutry C, Matsumoto H, Bos C et al. Decreased glutamate, glutamine and citrulline concentrations in plasma and muscle in endotoxemia cannot be reversed by glutamate or glutamine supplementation: a primary intestinal defect? Amino Acids 2012;43:1485-1498.

Perioperative arginine-supplemented nutrition in malnourished patients with head and neck cancer improves long-term survival

Buijs N
Van Bokhorst-de van der Schueren MA
Langius JA
Leemans CR
Kuik DJ
Vermeulen MA
Van Leeuwen PAM

8

Published in:
American Journal of Clinical Nutrition 2010; 92(5): 1151-1156.

abstract

Background: Plasma arginine concentrations are lower in patients with cancer, which indicates that arginine metabolism may be disturbed in these patients. Arginine supplementation has been associated with positive effects on anti-tumor mechanisms and has been shown to reduce tumor growth and to prolong survival. Furthermore, the prognosis of patients with head and neck cancer remains disappointing. Insufficient intake frequently leads to malnutrition, which contributes to high morbidity and mortality rates.

Objective: The aim of this study was to assess the long-term effects of perioperative arginine supplementation in severely malnourished patients with head and neck cancer.

Design: In this double-blind, randomized, controlled trial, we randomly assigned 32 severely malnourished patients with head and neck cancer to receive 1) standard perioperative enteral nutrition (n=15) or 2) arginine-supplemented perioperative enteral nutrition (n=17). The primary outcome was long-term (≥10 year) survival. Secondary outcomes included the long-term appearance of locoregional recurrence, distant metastases, and second primary tumors.

Results: No significant differences in baseline characteristics were observed between groups. The group receiving arginine-enriched nutrition had a significantly better overall survival (P=0.019) and better disease-specific survival (P=0.022). Furthermore, the arginine-supplemented group had a significantly better locoregional recurrence-free survival (P=0.027). No significant difference in the occurrence of distant metastases or occurrence of a second primary tumor was observed between the groups.

Conclusion: Perioperative arginine-enriched enteral nutrition significantly improved the long-term overall survival and long-term disease-specific survival in malnourished patients with head and neck cancer.

Introduction

Despite the available treatments, the survival of head and neck cancer patients remains disappointing because of uncontrollable persistent or recurrent disease. The incidence of postoperative complications within this population is 20–50%, which leads to prolonged hospital stays and a poorer prognosis. Malnutrition is one of the factors that contributes to disappointing morbidity rates. Most studies report malnutrition in 35–50% of all head and neck cancer patients, particularly in those with squamous cell carcinoma of the oropharyngeal and hypopharyngeal areas (1–6).

Plasma arginine concentrations are reduced in patients with cancer, which indicates that arginine metabolism is disturbed in the presence of a malignancy (7–10). Arginine-enriched nutrition in postoperative head and neck cancer patients is known to improve short-term outcomes, such as local wound complications, fistula rates, and length of hospital stays (11,12). Moreover, arginine supplementation is believed to augment specific and nonspecific anti-tumor mechanisms, such as retarding tumor growth and prolonging survival (13), whereby normal T-lymphocyte function is essential because of its substrate arginine (10). Actual prevention of malignant progression only manifests in the initiation and promotion phases. With the appearance of arginase, decreased T-cell proliferation, low expression of specific T-cell receptors, and decreased production of cytokines have been observed (14). Myeloid-derived suppressor cells appear to play a key role in these mechanisms by infiltrating the tumor and producing arginase, which enables the tumor to develop a cover shield against immune attacks (15).

In addition, arginine might play a role in carcinogenesis via its enzymatic conversion by nitric oxide synthase (NOS) with concomitant formation of nitric oxide (NO). Long-term exposure to low levels of NO induced by chronic inflammation may promote carcinogenesis by inhibiting apoptosis (8,16). In contrast, high levels of NO can be toxic for malignant cells (16,17). These tumor-toxic mechanisms particularly play a role in the initiation and promotion phases of the carcinogenesis. Generally, arginine and arginine-derived NO participate in many overlapping and conflicting regulatory processes, which lead to cancer development and prevention.

Our hypothesis is that, after surgical removal of malignant tumors, remnant cells can proliferate. This stage is comparable with the initiation and promotion

phases of carcinogenesis. In these circumstances arginine supplementation may improve T-cell function and promote NO production. The combination of these body defense mechanisms may combat remnant malignant cells. Thus, to prevent recurrence, arginine should be ideally administered perioperatively.

Until now, the long-term effects of arginine have never been explored. Therefore, the present study was designed to analyze the long-term (≥10 y) survival of malnourished patients undergoing head and neck surgery who received either arginine-enriched tube feeding or standard tube feeding. In this patient population, we studied the long-term overall survival, the long-term disease-specific survival, locoregional recurrence, distant metastases, second primary tumors, and the (possible) cause of death of head and neck cancer patients.

Subjects & Methods

Patients

In the period 1994–1997, 56 patients undergoing surgery for head and neck cancer who entered the Department of Otolaryngology/Head and Neck Surgery of the VU University Medical Center, Amsterdam, were studied. Patients who were severely malnourished, defined as a preoperative weight loss ≥10% over the past 6 months, were eligible for inclusion (18). The study was a prospective, randomized, double-blind, controlled clinical trial. All patients had a diagnosis of histologically proven squamous cell carcinoma of the oral cavity, larynx, oropharynx, or hypopharynx (18). Patients were excluded from the study if they received other investigational drugs or steroids; had renal insufficiency, hepatic failure, or any genetic immune disorder; or had a confirmed diagnosis of AIDS (18). The study was approved by the medical ethics committee of the VU University Medical Center (18).

This long-term survival study limited itself to 2 subgroups of patients: those who received standard enteral nutrition (control group) and those who received the arginine-enriched nutrition (arginine group) both preoperatively and postoperatively. The products were produced and blinded in the Netherlands (Nutricia Laboratories). An independent statistician generated the blinding procedure.

Nutrition

After stratification for type of surgery (combined mandibular resection or total laryngectomy) and previous radiotherapy (yes or no), the patients were randomly assigned to the arginine group or to the control group. The arginine group (n=17) received preoperative and postoperative enteral nutrition in which 41% of the casein was replaced by arginine, whereas the control group (n=15) received preoperative and postoperative enteral nutrition with a specifically formulated product that closely reflected the current standard of practice (standard formula) (Table1). Nutritional solutions were isocaloric and isonitrogenous. The only difference between the 2 was the amount of arginine. Specifically, no other immune-modulating nutrients (eg, omega-3 fatty acids, nucleotides, and antioxidants) were added.

Target intake was based on estimated energy requirements, calculated as 1.5 × the basal energy expenditure based on actual body weight. The patients were given their complete nutritional requirements by enteral feeding, but they were allowed to eat in addition to tube feeding on demand.

Postoperatively, all patients received the same tube feeding (1.5 × basal energy expenditure), either the arginine or the control product, starting on the first postoperative day until an X-ray conducted to assess swallowing ability performed 10 days after surgery showed no leakage from anastomoses. (Repeated "swallowing X-rays" were scheduled if anastomotic leakage occurred.) Patients were not allowed to eat next to their tube-feeding until 10 days after surgery. If patients were in need of prolonged tube-feeding after the 10th postoperative day, they were all given standard tube-feeding (ie, the special formula was not continued).

Event monitoring

The data were collected in August 2007, after a follow-up period of ≥10 years. The following events were monitored: survival or death, the occurrence of locoregional recurrence, the occurrence of distant metastases, and the occurrence of second primary tumors. The cause of death was categorized as follows: 0, alive; 1, in-hospital-death; 2, death from recurrent cancer; 3, death from second primary tumor; and 4, death other than cancer related. Causes 1, 2, and 3 were noted as disease-specific causes of death. Survival analyses were made for both overall survival and disease-specific survival. The variables were expressed in months after the date of surgery.

TABLE 1 | Composition per liter of the nutritional formulas[1].

	Supplement	
	Standard formula	**Arginine formula**
Protein (g)	62.50	36.85
Free arginine	0.00	12.50
Glutamine	6.30	3.70
Nitrogen	9.80	9.80
Fat (g)	48.61	48.61
Carbohydrate (g)	140.63	153.77
Energy (kJ)	5250	5250

[1] Assumptions: 1 g protein = 17 kJ, 1 g arginine = 17 kJ, 1 g carbohydrate = 17 kJ, 1 g fat = 38 kJ, glutamine content = 10% by weight of casein, conversion factor for casein = 6.38, and arginine contains 32.16% nitrogen.

Statistical procedures

Curves for overall survival, disease-specific survival, and disease-free survival were made according to the Kaplan-Meier method. Log-rank tests were used to compare survival between the control group and the arginine group. Cox regression was used to study confounding and effect modification. To bring the effect of arginine supplementation into perspective, crude hazard ratios (HRs) were computed with Cox regression and tabulated for the grouping variable and for several well-known risk factors. Calculations were made with SPSS version 16.0 (SPSS Inc, Chicago, IL) computer software. All P values <0.05 were considered to indicate statistical significance.

Results

Patients

Seventeen patients were randomly assigned to the arginine group and 15 patients to the control group. None of the patients dropped out after randomization. Preoperatively, all 32 patients were given enteral nutrition for 7–10 days through a nasogastric feeding tube. The groups did not differ significantly in age, sex, tumor stage, tumor localization, comorbidity, weight loss, type of operation, or type of reconstructive surgery (Table 2). In the preoperative period, patients in

the arginine group reached 113% and patients in the control group 110% of their estimated energy requirements, with 15% and 10% of intake originating from oral food intake, respectively. Preoperative tube feeding was provided for 8.6 ± 1.4 day (arginine group) and 8.8 ± 1.4 days (control group). Postoperatively, patients in the control group reached 96 ± 9% of their estimated requirements, whereas patients in the arginine group reached 83 ± 18% of their estimated requirements (NS). Overall, no difference in energy and protein intakes was observed between the groups, neither preoperatively nor postoperatively. For the patients in the arginine group, the mean total arginine intake over the preoperative and postoperative periods added up to 1015 ± 307 g—ie, 50 ± 15 g· d^{-1} or 0.72 ± 0.19 g · kg^{-1} · d^{-1}.

TABLE 2 | Baseline characteristics.

Characteristics	Arginine-supplemented group (n=17)	Control group (n=15)
Sex (male/female)	12/5	7/8
Age (y)	59 ± 12[1]	60 ± 8
Tumor stage (n)		
III	3	2
IVa	9	7
IVb	0	0
Recurrent tumor	5	5
Not staged	0	1
Tumor localization (n)		
Oral cavity	1	2
Larynx	3	3
Oropharynx	9	4
Hypopharynx	3	5
Other	1	1
Preoperative weight loss (%)	12.8 ± 5.1	17.1 ± 7.2

[1] Mean ± SD (all such values).

Long-term survival

All 15 patients in the control group and 14 of 17 patients in the arginine group had died at the time of data collection. Sixteen patients died of the consequences of recurrent cancer (locoregional recurrence and/or distant metastases), and 4 patients died because of a second primary tumor. Three patients died within the first 30 day after surgery (in-hospital death), and 6 patients died of causes other than cancer and were censored from the disease-specific survival analyses.

The median overall long-term survival was 34.8 mo in the arginine-supplemented group and 20.7 mo in the control group (P=0.019; Figure 1). Disease-specific survival was 94.4 mo in the arginine-supplemented group and 20.8 mo in the control group (P=0.022).

To exclude the influence of well-known, prognostic factors (TNM stage, margins, lymph nodes, preoperative weight loss, sex, and age) on survival, Cox regression analyses were performed to test for confounding and effect modification. N stage, and to a lesser extent weight loss and T stage, turned out to be confounders. When the confounders (either individually or combined) were entered into the model, the differences in survival between the arginine and control groups remained significant (P=0.031 with all 3 confounders in the model).

To put the effect of arginine supplementation into perspective, crude HRs were computed with Cox regression and tabulated for the grouping variable and for several well-known risk factors. The results for overall survival are shown in Table 3. Similar results were found for disease-free survival (not depicted in the table).

Being assigned to the arginine group had a positive influence on both overall survival (HR: 2.632; 95% CI: 1.142, 6.061) and disease-free survival (HR: 4.167; 95% CI: 1.389, 12.500). Absence of extracapsular spread had a positive effect on disease-free survival (HR: 2.8120; 95% CI: 1.008, 7.831) but not on overall survival. Lower tumor stages had marginally significant effects on overall survival, but these results attenuated disease-free survival. In the first instance, age was entered as a linear term in the model. However, because of the possibility on nonlinear effects, age was also entered as a nominal variable and a time-dependent covariate, which did not change the results.

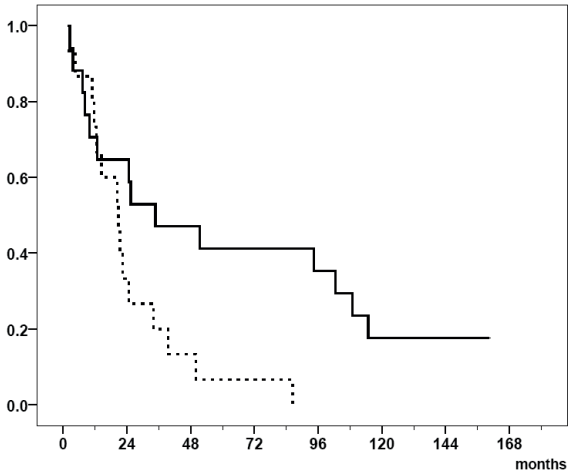

FIGURE 1 | Kaplan-Meier curve of the overall survival of severely malnourished patients with head and neck cancer after major surgery. The black line represents the arginine group (n=17), and the dotted line represents the control group (n=15).

Locoregional recurrence

Locoregional recurrence occurred in 4 of the 17 patients in the arginine group and in 9 of the 15 patients in the control group. Because less than half of the patients in the arginine group had died of locoregional recurrence, median time until development of locoregional recurrence could not be estimated, but was >92.8 mo; it was estimated at 10.6 mo in the control group (P=0.027; Figure 2). All patients with locoregional recurrence had died at the time of analysis; 9 of them were reported to have locoregional recurrence only, whereas another 4 were reported to have locoregional recurrence in combination with distant metastases. Locoregional recurrence-specific survival differed significantly between the 2 groups (P=0.010). When the confounders were accounted for, the observed effect became even more pronounced.

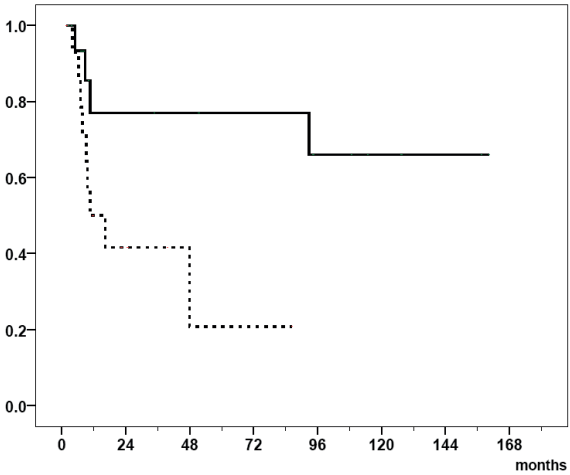

FIGURE 2 | Kaplan-Meier curve of locoregional recurrence-free survival in severely malnourished patients with head and neck cancer after major surgery. The black line represents the arginine group (n=17), and the dotted line represents the control group (n=15).

Distant metastases and second primary tumors

We observed no significant differences between either the development of distant metastases or the occurrence of second primary tumors between the arginine-supplemented and the control groups.

TABLE 3 | Hazard ratios (HRs) for the influence of perioperative variables on survival.

Perioperative variables	P	HR	95% CI
Group (arginine/control)	0.023	2.632	1.142, 6.061
Age	0.155	0.975	0.942, 1.010
Weight loss (%)	0.188	1.047	0.978, 1.120
Sex (male/female)	0.245	0.639	0.301, 1.360
Extracapsular spread (no/yes)	0.293	1.539	0.689, 3.440
Major postoperative complications (no/yes)	0.139	1.757	0.833, 3.705
Radical surgery (no/yes)	0.513	1.285	0.607, 2.270
N stage (≥N2)	0.552	1.256	0.593, 2.660
T stage (≥T4)	0.064	2.452	0.949, 6.336
Stage (≥IVa)	0.086	2.934	0.858, 10.037

Discussion

To our knowledge, our study was the first to show that a nutritional intervention with arginine-enriched nutrition before and after surgery may improve survival. Overall survival was a median of 34.8 mo in the arginine group and 20.7 mo in the control group; disease-specific survival was a median of 94.4 and 20.8 mo, respectively.

Despite the available surgical treatments, the survival of head and neck cancer patients remains disappointing because of uncontrollable persistent and recurrent disease. The most important prognostic factors are histologic tumor grade, clinical stage, tumor invasion, tumor thickness, angio-lymphatic invasion, and dissemination (19,20). Within our study, pathologic results concerning malignant tissue removed from the patients showed no significant differences in tumor stage and localization between groups. Furthermore, the type of resection and the type of reconstructive surgery were not significantly different between the 2 groups. Although suggested as prognostically important, the true predictable value is controversial.

Generally, in patients with cancer, immune suppression can be caused by malnutrition, surgical trauma, and the immunosuppressive capacity of the tumor itself. The immune suppression in these patients can be linked to low levels of arginine (15,18,21). Nutritional support with arginine is suggested to improve nutritional status and immune function, which may subsequently result in enhanced defense against tumor growth (22–25). Although the mechanisms by which our positive results can be explained remain speculative, interplay of arginine and immunologic defense seems plausible. Our hypothesis, advocating the concept of proliferating remnant malignant cells that could be attacked by arginine-induced improved T-cell function and enhanced NO production, originates from several pioneering studies (10,15,26). This hypothesis might also explain the difference in short-term survival and long-term outcome. Besides the large influence of direct postoperative effects, the primary arginine effect occurred during the supplementation phase, but the result was observed much later. First, the effects of arginine on NO metabolism have been observed in animal and human studies (27). Furthermore, increased concentrations of NO in the microenvironment have been noticed to induce cytostasis and cytotoxicity in tumor cells and to induce apoptosis by stimulating the tumor suppressor gene p53 (16,17,28,29). Recent data support the importance for this latter specific

action. In surgical patients with head and neck squamous cell carcinoma, some patients were shown to still possess p53 mutations in adjacent epithelial cells (30–32). Most head and neck squamous cell neoplasms are observed to develop within a field of premalignant cells, which show alterations associated with the process of carcinogenesis involving mutations in the p53 genes. Other recent studies provide additional similar evidence. After surgical cancer resection, part of the "field" may be left in the patient (33). Thus, arginine-derived NO could have activated the intact p53 genes and thereby switched on the immune system to clear this tissue from the residual normal-appearing cells (28,29). This may have been one of the key principles to explain better survival and lower the appearance of locoregional recurrence and longer recurrence-free survival in the arginine-supplemented group.

Second, arginine is essential for normal T-cell proliferation. T-cells play an important role in the body's defense against malignant cells. Rodriguez et al. showed decreased proliferation, a low expression of T-cell receptor CD3zeta chain, and a decreased production of cytokines in appearance of arginase, the enzyme that converts arginine (14). Moreover, high arginase activity surrounding the tumor has been suggested to host arginine metabolism, which subsequently leads to reduced arginine concentrations in cancer patients. These reduced arginine concentrations are observed independently of tumor type, tumor stage, and body mass index as investigated by Vissers et al (9).

Furthermore, arginase is produced by myeloid-derived suppressor cells (MDSC) (described as macrophages or immature dendritic cells) that are observed to deplete arginine and to impair T-cell proliferation by infiltrating the tumor (10,26,34). Different types of these myeloid cells are already detected in colon, lung, and renal cell tumors and could be well present in many other types of cancer (10,15,34). Interestingly, blocking arginase eliminated the suppressor function of the MDSC *in vitro* and even showed anti-tumor effects *in vivo*, because of normalized arginine metabolism. Arginine depletion could thus be a mechanism by which the tumor regulates the immune system in favor of its own survival. Therefore, immunotherapy could be of great value to restrict this mechanism, which could be accomplished by arginase blocking therapy or arginine administration and thereby improve arginine balance (10,35). However, no significant difference in the inflammatory response (lymphocytes, tumor necrosis factor-α, interleukin-6, and C-reactive protein concentrations) was observed in patients with head and neck cancer between an arginine-supplemented group and a control group (36).

Supplemental arginine has been administered in a variety of clinical trials. However, many studies have suggested that arginine can reduce tumor growth, and some have observed a negative influence on tumor growth. Nevertheless, the approach is controversial (37,38). New data showed that arginine can be converted into glutamine (39). On the basis of current knowledge, we advocate arginine supplementation in the initiation and promotion phases. Arginine should not be given in the subsequent (progression) phases of cancer, because it could stimulate angiogenesis and tumor growth through mechanisms involving NO and growth factors. A recent study by Ascierto et al. showed that reducing arginine concentrations in patients with metastatic melanoma may result in prolonged survival (40).

The benefit of arginine supplementation has been reported in several studies and has been registered in the European Society for Clinical Nutrition and Metabolism guidelines (41). Jones and Heyland (42) hypothesized that arginine supplementation could inhibit arginase and thus prevent dysfunction of the immune system. The significant better locoregional recurrence-free survival in the arginine group supports our hypothesis that arginine-derived NO and T-cells can provide surveillance against (pre)malignant cells after surgery.

Arginine was also shown to block the formation and development of colorectal tumors, as observed in restrained crypt cell hyperproliferation and enhanced expression of survivin (an inhibitor of apoptosis). This might be related to the increased NO concentrations and decreased arginine conversion by arginase (43). These results strongly support our hypothesis and clinical results.

As a result of the conflicting effects of arginine on cancer, a review by Edwards et al. reported the following: "Long term data regarding the impact of arginine supplementation on mortality are not available. It had been suggested that this is probably a result of persistent concern about a possible promotion of tumor growth in some cases." (37). Because of the long-term follow-up period, we were able to investigate the long-term effects of perioperative enteral arginine supplementation in head and neck cancer patients. Our study is innovative because it involved single nutrient supplementation, homogeneous patient groups, and the length of follow-up.

The long-term effects of arginine supplementation on survival in the current study should be considered promising; still, small numbers of patients were included, and contemplations with respect to future studies are inevitable. Postoperative factors, such as lifestyle (which includes exercise, a reduction in alcohol, and

healthy food) and malnutrition, may have influenced the results between the groups. However, this study was not powered and designed to adequately rule out these factors. Therefore, we cannot exclude that these confounding factors might have influenced the results.

However, this study inevitably suggests that arginine may be a potential new valuable player in the treatment of head and neck cancer patients: this apparently simple intervention is suggested to improve the prognosis of these patients. Larger studies are necessary to confirm our results and to disentangle the underlying mechanisms of the therapeutic activity of arginine on tumor cells and immune function.

In conclusion, this study suggests that arginine-enriched nutrition given perioperatively may be a valuable tool for improving long-term survival in malnourished head and neck cancer patients. Larger groups are needed to confirm these results.

REFERENCES

1. Brookes GB. Nutritional status—a prognostic indicator in head and neck cancer. Otolaryngol Head Neck Surg 1985;93:69–74.
2. Hussain M, Kish JA, Crane L, et al. The role of infection in the morbidity and mortality of patients with head and neck cancer undergoing multimodality therapy. Cancer 1991;67:716–721.
3. Preiser JC, Berre PJ, Van Gossum A, et al. Metabolic effects of arginine addition to the enteral feeding of critically ill patients. JPEN J Parenter Enteral Nutr 2001;25:182–187.
4. Sako K, Lore JM, Kaufman S, Razack MS, Bakamjian V, Reese P. Parenteral hyperalimentation in surgical patients with head and neck cancer: a randomized study. J Surg Oncol 1981;16:391–402.
5. Colasanto JM, Prasad P, Nash MA, Decker RH, Wilson LD. Nutritional support of patients undergoing radiation therapy for head and neck cancer. Oncology 2005;19:371–379.
6. Jager-Wittenaar H, Dijkstra PU, Vissink A, van der Laan BF, van Oort RP, Roodenburg JL. Critical weight loss in head and neck cancer—prevalence and risk factors at diagnosis: an explorative study. Support Care Cancer 2007;15:1045–1050.
7. Chiarla C, Giovannini I, Siegel JH. Plasma arginine correlations in trauma and sepsis. Amino Acids 2006;30:81–86.
8. Lind DS. Arginine and cancer. J Nutr 2004;134(suppl):2837S–2841S.
9. Vissers YLJ, Dejong CHC, Luiking YC, Fearon KCH, von Meyenfeldt MF, Deutz NEP. Plasma arginine concentrations are reduced in cancer patients: evidence for arginine deficiency? Am J Clin Nutr 2005;81:1142–1146.
10. Zea AH, Rodriguez PC, Atkins MB, et al. Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. Cancer Res 2005;65:3044–3048.
11. de Luis DA, Izaola O, Cuellar L, Terroba MC, Martin T, Aller R. Clinical and biochemical outcomes after a randomized trial with a high dose of enteral arginine formula in postsurgical head and neck cancer patients. Eur J Clin Nutr 2007;61:200–204.
12. de Luis DA, Izaola O, Cuellar L, Terroba MC, Martin T, Aller R. High dose of arginine enhanced enteral nutrition in postsurgical head and neck cancer patients. A randomized clinical trial. Eur Rev Med Pharmacol Sci 2009;13:279–283.
13. Stechmiller JK, Childress B, Porter T. Arginine immunonutrition in critically ill patients: a clinical dilemma. Am J Crit Care 2004;13:17–23.
14. Rodriguez PC, Quiceno DG, Ochoa AC. L-arginine availability regulates T-lymphocyte cell-cycle progression. Blood 2007;109:1568–1573.
15. Marx J. Cancer immunology. Cancer's bulwark against immune attack: MDS cells. Science 2008;319:154–156.
16. Dimmeler S, Haendeler J, Nehls M, Zeiher AM. Suppression of apoptosis by nitric oxide via inhibition of interleukin-1beta-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. J Exp Med 1997;185:601–607.
17. Bani D, Masini E, Bello MG, Bigazzi M, Sacchi TB. Relaxin activates the L-arginine-nitric oxide pathway in human breast cancer cells. Cancer Res 1995;55:5272–5275.
18. van Bokhorst-de van der Schueren MAE, Quak JJ, von Blombergvan der Flier BME, et al. Effect of perioperative nutrition, with and without arginine supplementation, on nutritional status, immune function, postoperative morbidity, and survival in severely malnourished head and neck cancer patients. Am J Clin Nutr 2001;73:323–332.

19. Kurokawa H, Zhang M, Matsumoto S, et al. The high prognostic value of the histologic grade at the deep invasive front of tongue squamous cell carcinoma. J Oral Pathol Med 2005;34:329–333.
20. Thomas GR, Nadiminti H, Regalado J. Molecular predictors of clinical outcome in patients with head and neck squamous cell carcinoma. Int J Exp Pathol 2005;86:347–363.
21. Popovic PJ, Zeh HJ, Ochoa JB. Arginine and immunity. J Nutr 2007;137(suppl 2):1681S–1686S.
22. Braga M, Gianotti L, Radaelli G, et al. Perioperative immunonutrition in patients undergoing cancer surgery: results of a randomized double-blind phase 3 trial. Arch Surg 1999;134:428–433.
23. Reilly JJ. Does nutrition management benefit the head and neck cancer patient? Oncology 1990;4:105–115.
24. Riso S, Aluffi P, Brugnani M, Farinetti F, Pia F, D'Andrea F. Postoperative enteral immunonutrition in head and neck cancer patients. Clin Nutr 2000;19:407–412.
25. van Bokhorst-de van der Schueren MAE, van Leeuwen PA, Kuik DJ, et al. The impact of nutritional status on the prognoses of patients with advanced head and neck cancer. Cancer 1999;86:519–527.
26. Bronte V, Serafini P, Mazzoni A, Segal DM, Zanovello P. L-arginine metabolism in myeloid cells controls T-lymphocyte functions. Trends Immunol 2003;24:302–306.
27. Vermeulen MAR, van de Poll MCG, Ligthart-Melis GC, et al. Specific amino acids in the critically ill patient–exogenous glutamine/arginine: a common denominator? Crit Care Med 2007;35(suppl):S568–576.
28. Messmer UK, Ankarcrona M, Nicotera P, Brune B. p53 expression in nitric oxide-induced apoptosis. FEBS Lett 1994;355:23–26.
29. Nicotera P, Bonfoco E, Brune B. Mechanisms for nitric oxide-induced cell death: involvement of apoptosis. Adv Neuroimmunol 1995;5:411–420.
30. Brennan JA, Mao L, Hruban RH, et al. Molecular assessment of histopathological staging in squamous-cell carcinoma of the head and neck. N Engl J Med 1995;332:429–435.
31. Liao CT, Chang JT-C, Wang HM, et al. Analysis of risk factors of predictive local tumor control in oral cavity cancer. Ann Surg Oncol 2008;15:915–922.
32. van Houten VM, Tabor MP, van den Brekel MW, et al. Mutated p53 as a molecular marker for the diagnosis of head and neck cancer. J Pathol 2002;198:476–486.
33. Tabor MP, Brakenhoff RH, van Houten V, et al. Persistence of genetically altered fields in head and neck cancer patients: biological and clinical implications. Clin Cancer Res 2001;7:1523–1532.
34. Kusmartsev S, Gabrilovich DI. Role of immature myeloid cells in mechanisms of immune evasion in cancer. Cancer Immunol Immunother 2006;55:237–245.
35. Capuano G, Rigamonti N, Grioni M, Freschi M, Bellone M. Modulators of arginine metabolism support cancer immunosurveillance. BMC Immunol 2009;10:1.
36. de Luis DA, Arranz M, Aller R, Izaola O, Cuellar L, Terroba MC. Immunoenhanced enteral nutrition, effect on inflammatory markers in head and neck cancer patients. Eur J Clin Nutr 2005;59:145–147.
37. Edwards PD, Topping D, Kontaridis MI, Moldawer LL, Copeland EM, Lind DS. Arginine-enhanced enteral nutrition augments the growth of a nitric oxide-producing tumor. JPEN J Parenter Enteral Nutr 1997;21:215–219.

38. Park KG, Heys SD, Blessing K, et al. Stimulation of human breast cancers by dietary L-arginine. Clin Sci (Lond) 1992;82:413–741.

39. Loi C, Zazzo JF, Delpierre E, et al. Increasing plasma glutamine in postoperative patients fed an arginine-rich immune-enhancing diet—a pharmacokinetic randomized controlled study. Crit Care Med 2009;37:501–509.

40. Ascierto PA, Scala S, Castello G, et al. Pegylated arginine deiminase treatment of patients with metastatic melanoma: results from phase I and II studies. J Clin Oncol 2005;23:7660–7668.

41. Weimann A, Braga M, Harsanyi L, et al. ESPEN guidelines on enteral nutrition: surgery including organ transplantation. Clin Nutr 2006;25:224–244.

42. Jones NE, Heyland DK. Pharmaconutrition: a new emerging paradigm. Curr Opin Gastroenterol 2008;24:215–222.

43. Ma Q, Wang Y, Gao X, Ma Z, Song Z. L-arginine reduces cell proliferation and ornithine decarboxylase activity in patients with colorectal adenoma and adenocarcinoma. Clin Cancer Res 2007;13:7407–7412.

A new key player in VEGF-dependent angiogenesis in human hepatocellular carcinoma: dimethylarginine dimethylaminohydrolase 1

Buijs N
Oosterink JE
Jessup M
Schierbeek H
Wisselink W
Stolz DB
Houdijk APJ
Geller DA
Van Leeuwen PAM

Published in:
Angiogenesis 2017; 20(4): 557-565.

abstract

Background: Anti-angiogenic therapies, targeting VEGF, are a promising treatment for hepatocellular carcinoma (HCC). To enhance this potential therapy, identification of novel targets in this pathway is of major interest. Nitric oxide (NO) plays a crucial role in VEGF-dependent angiogenesis. NO production depends on arginine as substrate and asymmetric dimethylarginine (ADMA) as inhibitor. Dimethylarginine dimethylaminohydrolase 1(DDAH-1) catabolizes ADMA and therefore regulates NO and VEGF expression. This study unravels additional mechanisms to improve VEGF targeting therapies.

Methods: The expression of DDAH-1 was examined in HCC specimen and non-tumorous background liver of 20 patients undergoing liver resection. Subsequently, arginine/ADMA balance, NO production, and VEGF expression were analyzed. The influence of hypoxia on DDAH-1 and angiogenesis promoting factors was evaluated in HepG2 cells and primary human hepatocytes.

Results: DDAH-1 expression was significantly induced in primary HCC tumors compared to non-tumorous background liver. This was associated with an increased arginine/ADMA ratio, higher NO formation, and higher VEGF expression in human HCC compared to non-tumorous liver. Hypoxia induced DDAH-1, iNOS, and VEGF expression in a time-dependent manner in HepG2 cells.

Conclusions: Our results indicate that DDAH-1 expression is increased in human HCC, which is associated with an increase in the arginine/ADMA ratio and enhanced NO formation. Hypoxia may be an initiating factor for the increase in DDAH-1 expression. DDAH-1 expression is associated with promotion of angiogenesis stimulating factor VEGF. Together, our findings for the first time identified DDAH-1 as a key player in the regulation of angiogenesis in human HCC, and by understanding this mechanism, future therapeutic strategies targeting VEGF can be improved.

Introduction

Hepatocellular carcinoma (HCC) is a global health problem, representing the fifth most common cancer and the third most common cause of death from cancer worldwide. The incidence of this primary liver cancer has increased in the last decades (1). Clarifying the underlying mechanisms responsible for HCC development and progression may lead to the identification of key targets for therapeutic intervention.

Hepatocarcinogenesis is a multistep process involving genetic and environmental changes, which allow the hepatocyte to escape normal control mechanisms in cell proliferation, differentiation, migration, and death, resulting in the evolvement of malignant disease (2). Angiogenesis is a crucial process in HCC development since HCC is one of the most vascular solid tumors known. Vascular endothelial growth factor (VEGF) is the primary mediator of angiogenesis in primary liver tumors (3). Anti-angiogenic therapies, targeting VEGF, such as sorefenib (blockage of the VEGF tyrosine kinase receptor) and bevacizumab (antibody to VEGF), have been shown to be a promising effective treatment for advanced HCC, and sorafenib has now been approved for the treatment of advanced HCC in both the USA and Europe (4,5). To enhance this type of targeted treatment for HCC, evaluating the effect of vertical blockade in which the VEGF pathway is interrupted at different points, is needed. This concept is appealing because it may lead to more complete blockade, block feedback loops, and have non-overlapping resistance patterns (6). Therefore, the identification of novel targets in this pathway is of major interest.

Nitric oxide (NO), produced from arginine by the nitric oxide synthases (NOS), is a crucial molecule and regulator of angiogenesis. NO enhances the expression of other angiogenic factors, vascular permeability, perivascular cell recruitment, and vessel remodeling and maturation (7). NO has a reciprocal role in VEGF mediated angiogenesis by stimulating the expression of VEGF and mediating its downstream angiogenic effects.

Regulation of NO production may therefore be crucial in the regulation of VEGF-dependent angiogenesis and consequently tumor progression. The amino acid arginine is the sole precursor for NO via the three isoforms of NOS: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) (8). Asymmetric dimethylarginine (ADMA) is a competitive inhibitor of all NOS isoforms (9). The ratio between arginine and ADMA is the indicator for NOS activity and therefore NO formation. ADMA is predominantly catalyzed by the enzyme dimethylarginine

dimethylaminohydrolase (DDAH) (10). Thus, ADMA and DDAH have an important regulatory role in NO production, and the balance between arginine and ADMA (arginine/ADMA ratio) is the preserving factor in this pathway (7,11). The DDAH enzyme has two isoforms: DDAH-1 is the most important isoform in ADMA metabolism, and the liver is a major expression site. DDAH-2 is particularly expressed in vascular tissue, and it was found that ADMA levels do not evidently depend on DDAH-2 activity (12).

The role of ADMA and the DDAH isoforms in angiogenesis is excessively studied in cardiovascular settings. It was shown that ADMA and DDAH-1 metabolism significantly influences NO formation and hereby plays a role in the regulation of endothelial function, vascular condition and development (13-15). Moreover, increased DDAH-1 expression is associated with upregulation of angiogenic factors, most importantly VEGF (16). These findings suggest that DDAH-1 and ADMA may be potential anti-angiogenic targets in cancer treatment. Insights in the mechanisms of action of these players in HCC development may provide new pointers in treatment and amplify the battle against this malignancy.

We hypothesized that DDAH-1 may be a potential target in HCC therapy. Therefore, we evaluated whether DDAH-1 expression is increased in HCC tissue compared to non-cancerous liver tissue of 20 patients who underwent hepatic resection. In the same tissue samples, we determined the arginine/ADMA ratio, NO formation, and VEGF expression. In addition, we determined the effect of hypoxia on DDAH-1, iNOS, and VEGF expression in HCC cells.

Materials & Methods

In vivo materials

Tissue and serum samples were collected from 20 HCC patients undergoing surgical resection at the Liver Cancer Center of the University of Pittsburgh Medical Center under an institutional review board (IRB)-approved protocol. From each patient, a HCC sample, a non-tumorous liver sample, and serum were collected. Serum of these patients undergoing hepatic resection for HCC (n=20) and from a control group of patients undergoing hepatic resection for benign lesions (n=10) was collected preoperatively. Patient tissue samples and serums were stored at −80 °C until analysis. Informed consent was obtained from all individual participants included in the study.

In vitro materials

Experiments were also done in cell cultures. The HepG2 cell line (HB-8065) was purchased from and tested by American Type Culture Collection (Manassas, VA), and the primary hepatocytes were obtained from histologically normal liver under an IRB-approved protocol.

Immunoblotting analysis

Whole-cell protein was extracted from the HCC and corresponding non-tumorous tissue samples with tissue protein extraction reagent (Pierce Biotechnology, Rockford, IL). Whole-cell protein of the cultured primary hepatocytes and HepG2 cells were extracted with cell lysis reagent (Sigma, St. Louis, MO). Samples were quantified, and 20–100μg of total protein was separated by gel electrophoresis. The separated proteins were transferred to a membrane, and membranes were blocked in 5% low-fat milk, and western blotting was performed with antibodies against DDAH-1 (Santa Cruz Biotechnology Inc., Dallas, TX) (1:500), iNOS (BD Biosciences, San Jose, CA) (1:500), VEGF (Santa Cruz Biotechnology Inc., Dallas, TX) (1:500), and β-actin (Abcam, Cambridge, MA) (1:1000) diluted in 1% low-fat milk solutions overnight. The membranes were washed with Tris-buffered saline with Tween (TBST) and incubated with horseradish peroxidase-conjugated secondary antibodies and washed again with TBST. Chemiluminescent substrate (Thermo Fisher Scientific, Rockford, IL) was added to the membranes for 3–7 minutes. Excessive substrate was removed, and the membranes were placed in transparent plastic sheets and exposed to film (Laboratory Product Sales Inc, Rochester, NY).

Immunofluorescence

HCC tissue samples and non-tumorous liver tissue samples were fixed in 2% paraformaldehyde for 2 h and placed in 30% sucrose for 24 h. The fixed tissue samples were placed in liquid nitrogen cooled 2-methylbutane, and cryostat sections (6–10 μm) were placed onto glass slides. After rehydration with phosphate-buffered saline, the tissue sections were permeabilized with 0.1% Triton X-100 (Sigma, St. Louis, MO) and washed in protein blocking buffer (PBB; 0.5% bovine serum albumin in phosphate-buffered saline) after each treatment. Tissue sections were blocked in the serum of the host of the secondary antibody for 45 minutes at room temperature (Sigma, St. Louis, MO; Millipore, Billerica, MA). After 1-h incubation with specific primary antibody for DDAH-1(Santa Cruz

Biotechnology Inc., Dallas, TX) (1:100 dilution in PBB with 0.1% triton X-100), iNOS (BD Biosciences, San Jose, CA) (1:100 dilution in PBB), and VEGF (Abcam, Cambridge, MA) (1:250 dilution in PBB) at room temperature, tissue sections were treated with species specific secondary antibody and fluor-conjugated phalloidin (Invitrogen, Carlsbad, CA) for 1 h at room temperature. Hoechst stain was added for 30 s. Fluorescent images were captured with a confocal microscope (FluoView 1000, Olympus, Tokyo, Japan). Quantification analyses were performed by means of traditional binary threshold using the negative staining control as a reference point for staining intensity of an area per nucleus.

Arginine and ADMA measurements

The arginine/ADMA ratio was determined by measuring arginine and ADMA concentrations by using a previously described liquid chromatography mass spectrometry method (17).

Serum and tissue NO assay

NO in serum and tissue was predicted by measuring nitric oxide metabolites (NOx) by the Griess reaction after conversion of nitrate to nitrite using a nitric oxide colorimetric assay kit (Cayman Chemical Company, Ann Arbor, MI). The NOx measurements were performed in preoperative serum samples of the HCC patients (n=20); the serum of patients with benign liver lesions functioned as control (n=10). Also, tissue homogenates of 20 paired HCC samples, and their non-tumor counterparts were analyzed. Serum and tissue samples preparation was according to the manufacturer's protocol. NOx concentrations were defined as μM per μg protein in tissue homogenates and as μM per L serum.

Hypoxia experiment

HepG2 cells were seeded on 12 sterile culture dishes. After 24 h of incubation, the cells were washed and incubated under normoxic (21.0% O_2) and hypoxic (1.0% O_2) conditions in duplicate for 0, 1, 3, 6, 12, and 24 h. DDAH-1, iNOS, and VEGF expressions were determined by immunoblotting as described above.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using the Student's *t*-test. SPSS 20.0 for Windows (SPSS Inc., Chicago, IL) was used for statistical analysis. $P < 0.05$ was considered as statistically significant.

TABLE 1 |

Clinicopathological features	Value
Number of patients	20
Sex	8 female; 12 male
Age (years)	66 ± 3
Cirrhosis	5 (25%)
Hepatitis	
HBV	1 (5%)
HCV	5 (25%)
Non-viral Hepatitis	
ETOH	2 (10%)
NASH	8 (40%)
Other	1 (5%)
Mean Tumor Size (cm)	6.8 ± 1
No. Tumor Nodules	
1	10 (50%)
≥2	10 (50%)
Differentiation	
Well	4 (20%)
Moderate	16 (80%)
Poor	0 (0%)
Vascular invasion	11 (55%)
Stage	
I	5 (25%)
II	15 (75%)
III	5 (25%)

HBV, hepatitis B virus. HCV, hepatitis C virus. ETOH, alcohol induced hepatitis. NASH, non-alcoholic steatohepatitis.

Results

Patient characteristics are shown in Table 1. We found a higher expression of DDAH-1 in the HCC tumor samples compared to the corresponding non-tumorous liver tissue samples (Figure 1A). Immunofluorescence confirmed increased DDAH-1 expression in the primary HCC tumors compared to non-tumorous liver tissue. DDAH-1 was localized in hepatocytes and most abundant in HCC cells, whereas expression of DDAH-1 in endothelial cells of vascular structures was not observed (Figure 1B). *In vitro* experiments also showed that the expression of DDAH-1 was increased in the HCC cell line HepG2 compared to normal primary hepatocytes (Figure 1C).

To determine the effect of the observed increase in DDAH-1 expression on the arginine/ADMA ratio (the indicator for NO production), arginine and ADMA concentrations in HCC and the paired non-tumorous tissue of 20 patients were measured using mass spectrometry. A higher arginine/ADMA ratio is linked to a higher NO production. The arginine/ADMA ratio was 74% higher in HCC tissue compared to the non-tumorous liver tissue (137 ± 29 vs. 79 ± 7, respectively) (Figure 2).

To study the effect of the increased DDAH-1 expression and subsequently increased arginine/ADMA ratio in HCC on NO formation in these patients, NO metabolites were measured in the same tissue homogenates. We found significant higher NOx levels in the HCC homogenates compared to the non-tumorous liver homogenates (Figure 3A). Furthermore, NOx levels in serum of HCC patients were significantly higher compared to non-cancer patients (Figure 3B).

We also found that the increased DDAH-1 expression in HCC tissue was accompanied by an increased expression of the angiogenesis stimulating factor VEGF (Figure 4A). *In vitro*, the HCC cell line also showed an increased VEGF and iNOS expression compared to the primary hepatocytes (Figure 4B, C).

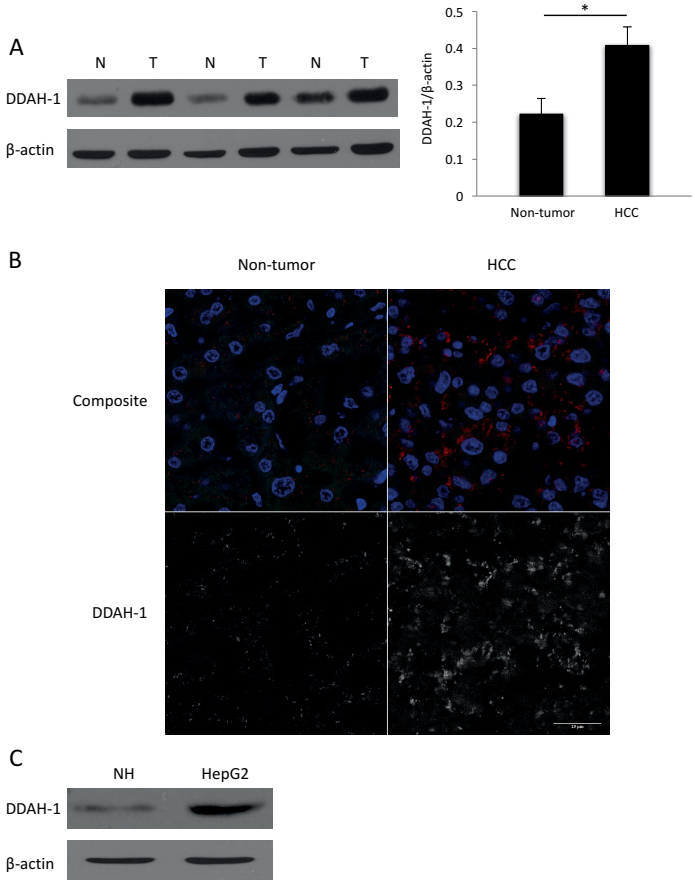

FIGURE 1 | Overexpression of DDAH-1 in HCC. **A** DDAH-1 protein levels were measured with immunoblotting in paired HCC samples and their non-tumorous counterparts. Protein expression results were normalized to internal control β-actin. *P < 0.05. N non- tumorous liver (n=20), T HCC tumor (n=20). Imagings shown are representative results of three patients. **B** Non- tumorous human liver and human HCC tissues were stained for DDAH-1. In the composite images: Red, DDAH- 1; blue, nuclei. In the single DDAH-1 channel images: DDAH-1, gray. **C** Expression of DDAH-1 in *in vitro* cultured human primary hepatocytes and in a HCC cell line was detected with immunoblotting analysis.

Hypoxia is often seen in solid tumors, including primary liver tumors. This is confirmed in a recent study by our group, showing that hypoxia-inducible factor-1 alpha (HIF-1α) is indeed substantially increased in HCC specimens, compared to the non-tumorous specimens of our patients (18). Hypoxia and the subsequent expression of HIF-1α have shown to induce the expression of iNOS and VEGF (19,20). To determine whether tumor hypoxia also influences the expression of DDAH-1, we performed this *in vitro* experiment: HepG2 cells were cultured under normoxic and hypoxic conditions. DDAH-1 expression increased in hypoxia in a time-dependent manner. The expression of the angiogenesis promoting factors iNOS and VEGF showed a similar increase in time in the hypoxic HepG2 cells (Figure 5).

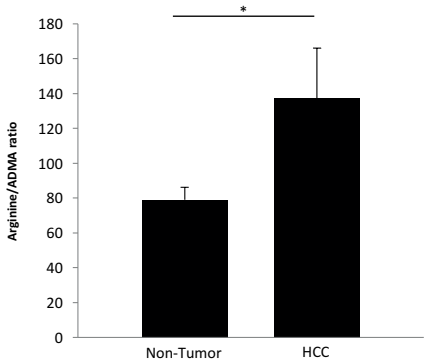

FIGURE 2 | Increased arginine/ADMA ratio in HCC tissue. Arginine and ADMA concentrations in HCC tissue (n=20) and non-tumorous liver tissue (n=20) were measured with a liquid chromatography mass spectrometry method. *P <0.05.

Discussion

Despite advances in surgical and ablative techniques in the past decades, HCC is still a leading cause of cancer-related death worldwide. Many patients are diagnosed with HCC in an advanced state of malignant disease, and there remains a lack of effective chemotherapeutic treatment for widely progressive disease (21,22). Recently the therapeutic application of anti-angiogenetic agents in HCC, targeting the VEGF pathway, has shown promising results in the treatment of advanced HCC. The search for novel targets in the VEGF pathway in HCC to complement this therapy may clarify molecular and metabolic changes

in hepatocarcinogenesis and improve therapeutic effects. Therefore, obtaining more insight in these mechanisms of action is an important objective in advance of novel strategies to prevent and treat primary liver tumors.

With regard to our results, we hypothesize on the HCC-induced mechanisms to allow malignant outgrowth: Solid tumors create an environment which prevents the immune system from counteracting tumor development and promote malignant progression, indicating tumor sprouting and angiogenesis. NO augments DNA synthesis, cell proliferation, and migration and mediate the function of multiple angiogenetic factors, such as VEGF, and hence NO is essential for tumor progression. HCC cells stimulate the formation of iNOS-derived NO by the expression of cytokines, e.g., tumor necrosis factor alpha (TNF-α) and HIF-1α. The arginine/ADMA ratio is the preserving factor in NO synthesis, since arginine is its sole precursor and ADMA is the competitive antagonist. DDAH catabolizes ADMA, and therefore, enhanced DDAH activity results in an increase in NO formation. Enhanced DDAH expression could be a mediating step in the pathway of NO synthesis induced by tumor cytokine activation to promote tumor growth. Our study shows a clear association between DDAH-1 expression, arginine/ADMA ratio, and subsequently NO formation and VEGF expression in human HCC.

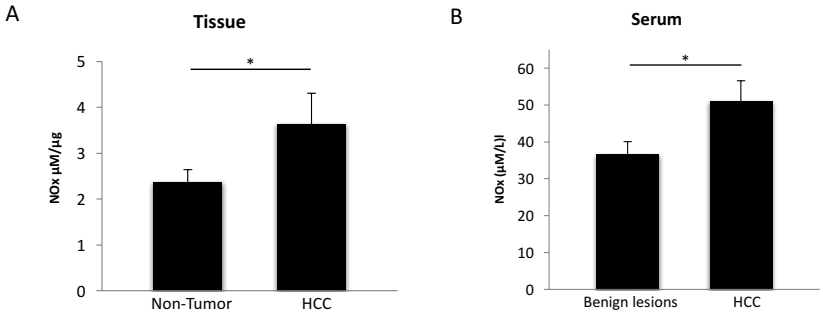

FIGURE 3 | NO metabolites were increased in HCC tissue (n=20) compared to non-tumorous liver tissue (n=20) and were higher in serum of HCC patients (n=20) compared to serum of patients with benign lesions (n=10). NO metabolites were analyzed in tissue (**A**) and serum (**B**) by using a Griess reagent protocol. NOx concentration was defined as µM per µg protein in tissue homogenates and as µM per L serum. *P<0.05.

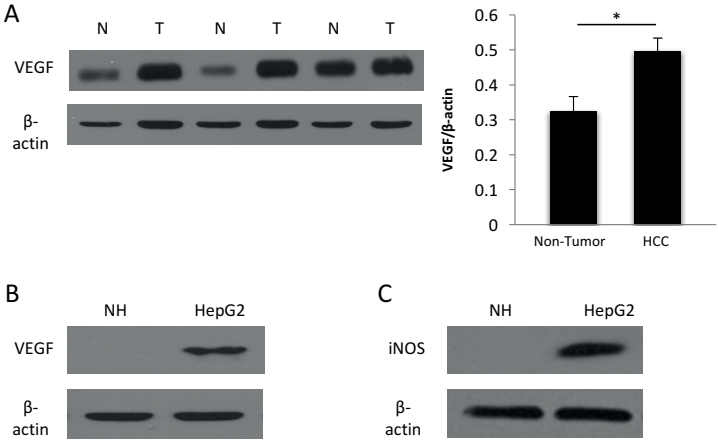

FIGURE 4 | Overexpression of angiogenesis promoting factors VEGF and iNOS in HCC. (**A)** VEGF protein levels were measured with immunoblotting analysis in paired HCC samples (n=20) and their non-tumorous counterparts (n=20). Protein expression results were normalized to internal control β-actin. *P < 0.05. N non-tumorous liver, T tumor. Expression of VEGF (**B**) and iNOS (**C**) in *in vitro* human primary hepatocytes and in a HCC cell line were detected with immunoblotting analysis.

One of the most prominent factors implicated in angiogenesis and tumor progression is VEGF. This angiogenetic mediator induces vascular sprouting, increases endothelial permeability, and maintains vascular integrity in the tumor (23). NO derived from tumor-induced iNOS expression is also a key regulator of angiogenesis and tumor growth. This inducible isoform of NOS is only expressed in stressed tissue. Whereas nNOS and eNOS produce small amounts of NO in a pulsatile manner, iNOS continuously produces high amounts of NO (24).

NO induces the expression of VEGF and mediates its angiogenesis stimulating effects. VEGF on its turn stimulates the expression of iNOS and its continuous production of NO. Furthermore, the expressions of iNOS and VEGF are closely related to tumor angiogenesis and are involved in tumor metastasis and invasiveness (25,26).

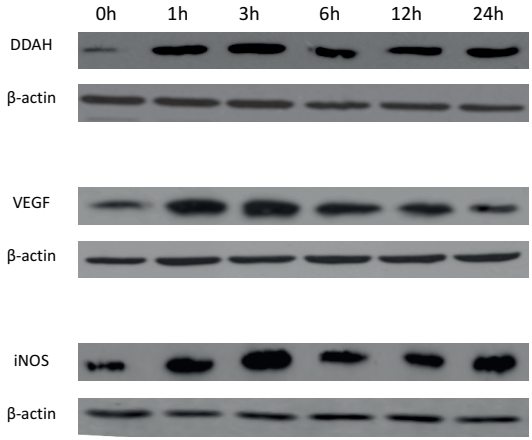

FIGURE 5 | Hypoxia induces overexpression of DDAH-1, VEGF and iNOS in HCC cells in a time-dependent manner. Expression of DDAH-1, VEGF and iNOS was determined by immunoblotting in HepG2 cells subjected to a time course up to 24 h of hypoxia (1% O_2).

Arginine is used by all NOS isoforms to form NO. ADMA regulates this NO production by competing with arginine for NOS and therefore blocking the formation of NO. NOS is mainly localized in the cell, and thus the intracellular ADMA and arginine levels influence NO production. Extracellular ADMA is also an antagonist to extracellular arginine on cell membrane transporter level, whereas they are both transported into the cell via cationic amino acid transporters of system y+ (27,28). Thus, NO production depends on the arginine/ADMA ratio, which was also shown in our results.

ADMA is catabolized by DDAH and the high DDAH-1 expression as found in our human HCC specimens was associated with an increase in the arginine/ADMA ratio and higher NO formation. Although the role of both arginine and NO are excessively studied in the oncological setting, studies on the role of ADMA and DDAH in human tumor development and progression are lacking. Though, ADMA and DDAH are widely analyzed in the cardiovascular setting as regulators of NO dependent endothelial function, vascular tone, organ perfusion, and vascular proliferation (16). Low DDAH activity and subsequently increased ADMA levels cause a deficiency in NO bioavailability, and this results in cardiovascular dysfunction. There are only a few studies that translated the role of ADMA and DDAH in the oncological setting. Those studies reported that both isoforms of DDAH indeed may play a role in the development of tumor vasculature (29).

A key role for DDAH in tumor angiogenesis is supported by studies showing that DDAH overexpression activates angiogenic pathways *in vitro*. Vanella et al. showed that DDAH-2, iNOS, and VEGF expressions were higher in a prostate cancer cell line compared to cells that represent benign prostate hypertrophy (30). Consistent to our results, Kostourou et al. showed in *in vivo* experiments in rats bearing glioma xenografts that overexpression of DDAH-1 and a subsequent decreased inhibiting effect of ADMA on NOS result in increased tumor growth, tumor vascularization, and VEGF secretion (31). We now report that DDAH-1 is overexpressed in human HCC compared to non-tumorous liver, that this increase in DDAH-1 expression results in enhanced NO formation and is associated with stimulation of angiogenetic factors. More studies are needed to further unravel the mechanisms behind this pathway; for example, DDAH-1 knockdown experiments in rodents may gain more insight into the point of action of DDAH-1 in VEGF-dependent angiogenesis and tumor growth of HCC.

HCC shows signs of hypoxia, which is associated with tumor progression and a poor prognosis. Increased expression of angiogenesis promoting factors is required for tumor growth, counteracting cancer cell hypoxia (32). However, the mechanism by which deprivation of adequate oxygen supply influences cancer progression is still unclear. Previous studies showed that NO production is primarily present in tumor areas between viable and necrotic tumor regions, the hypoxic area (33). Hypoxic tissue induces the expression of hypoxia-inducible factor-1 alpha (HIF-1α), which stimulates the expression of iNOS and VEGF, which was also seen in our hypoxia experiment in the HCC cells. In the clinical setting, malignancies with high iNOS and VEGF expression typically present as highly vascularized tumors (34). When inhibiting iNOS and VEGF activity in tumors, the tumor vasculature becomes dysfunctional and tumor perfusion is not effective, resulting in decreased tumor growth (35,36). Our study also aimed to determine the effect of tumor hypoxia on DDAH-1 expression and the arginine/NO pathway. We found that hypoxia also induces DDAH-1 expression in HCC cells.

Our results suggest that DDAH has a regulating role in tumor angiogenesis in humans by stimulating NO formation and VEGF expression. Multiple trials already studied the clinical effects of angiogenesis targeted therapy (37,38). Incomplete knowledge and complexity of the underlying mechanisms of the hepatocarcinogenesis are most likely to be the restrictive factors in refining these therapies (39). The results of our study further elucidate the complex tumor-promoting pathways in HCC. Whereas VEGF inhibitors alone show promising

effects in the treatment of HCC, our study suggests an additional novel role for DDAH-targeted therapy in HCC to significantly improve clinical outcome in HCC patients. As stated above, tumors with high DDAH-1 expression grow almost twice as fast as controls, which clearly shows the importance of DDAH in tumor progression (31). Combined with our results, inhibiting DDAH may serve as a potential anti-tumor strategy. Therefore, we hypothesize the development of multiple-targeted inhibitors to control NO formation and subsequently angiogenesis and tumor progression.

In conclusion, our data show that DDAH-1 is overexpressed in HCC and plays a pivotal role in the regulation of hypoxia-induced angiogenesis by regulating the NO/VEGF pathway. These findings suggest that DDAH-1 may serve as a promising target for development of novel therapeutic agents for HCC and that future studies are needed to further explore the role of DDAH-1 in the hepatocarcinogenesis and as a targeted agent in HCC therapy development.

References

1. Rampone B, Schiavone B, Confuorto G. Current management of hepatocellular cancer. Curr Oncol Rep 2010;12(3):186-92.
2. Hernandez-Gea V, Toffanin S, Friedman SL, Llovet JM. Role of the microenvironment in the pathogenesis and treatment of hepatocellular carcinoma. Gastroenterology 2013;144(3):512-27.
3. Moon WS, Rhyu KH, Kang MJ, et al. Overexpression of VEGF and angiopoietin 2: a key to high vascularity of hepatocellular carcinoma? Mod Pathol 2003;16(6):552-7.
4. Fang P, Hu JH, Cheng ZG, Liu ZF, Wang JL, Jiao SC. Efficacy and safety of bevacizumab for the treatment of advanced hepatocellular carcinoma: a systematic review of phase II trials. PLoS One 2012;7(12):e49717.
5. Llovet JM, Ricci S, Mazzaferro V, et al. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 2008;359(4):378-90.
6. Siegel AB, Olsen SK, Magun A, Brown RS, Jr. Sorafenib: where do we go from here? Hepatology 2010;52(1):360-9.
7. Ma Q, Wang Z, Zhang M, et al. Targeting the L-arginine-nitric oxide pathway for cancer treatment. Curr Pham Des 2010;16(4):392-410.
8. Luiking YC, ten Have GA, Wolfe RR, Deutz NE. Arginine *de novo* and nitric oxide production in disease states. Am J Physiol Endocrinol Metab 2012;303(10):E1177-89.
9. Vallance P, Leone A, Calver A, Collier J, Moncada S. Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. Lancet 1992;339(8793):572-5.
10. Boger RH. Asymmetric dimethylarginine, an endogenous inhibitor of nitric oxide synthase, explains the "L-arginine paradox" and acts as a novel cardiovascular risk factor. J Nutr 2004;134(10 Suppl):2842S-47S.
11. Fiedler LR, Wojciak-Stothard B. The DDAH/ADMA pathway in the control of endothelial cell migration and angiogenesis. Biochem Soc Trans 2009;37(Pt 6):1243-7.
12. Pope AJ, Karrupiah K, Kearns PN, Xia Y, Cardounel AJ. Role of dimethylarginine dimethylaminohydrolases in the regulation of endothelial nitric oxide production. J Biol Chem 2009;284(51):35338–35347.
13. Siegerink B, Maas R, Vossen CY, et al. Asymmetric and symmetric dimethylarginine and risk of secondary cardiovascular disease events and mortality in patients with stable coronary heart disease: the KAROLA follow-up study. Clin Res Cardiol 2013;102(3):193–202.
14. Anderssohn M, Rosenberg M, Schwedhelm E, et al. The l-Arginine–asymmetric dimethylarginine ratio is an independent predictor of mortality in dilated cardiomyopathy. J Card Fail 2012;18(12):904–911.
15. Ghebremariam YT, Erlanson DA, Cooke JP. A novel and potent inhibitor of dimethylarginine dimethylaminohydrolase: a modulator of cardiovascular nitric oxide. J Pharmacol Exp Ther 2014;348(1):69–76.
16. Fiedler LR, Bachetti T, Leiper J, et al. The ADMA/DDAH pathway regulates VEGF-mediated angiogenesis. Arterioscler Thromb Vasc Biol 2009;29(12):2117–2124.

17. Davids M, Swieringa E, Palm F, et al. Simultaneous determination of asymmetric and symmetric dimethylarginine, L-monomethylarginine, l-arginine, and L-homoarginine in biological samples using stable isotope dilution liquid chromatography tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2012;900:38–47.
18. Yan W, Chang Y, Liang X, et al. High-mobility group box 1 activates caspase-1 and promotes hepatocellular carcinoma invasiveness and metastases. Hepatology 2012;55(6):1863–1875.
19. Kimura S, Kitadai Y, Tanaka S, et al. Expression of hypoxia-inducible factor (HIF)-1alpha is associated with vascular endothelial growth factor expression and tumour angiogenesis in human oesophageal squamous cell carcinoma. Eur J Cancer 2004;40(12):1904–1912.
20. Takala H, Saarnio J, Wiik H, Ohtonen P, Soini Y. HIF-1alpha and VEGF are associated with disease progression in esophageal carcinoma. J Surg Res 2011;167(1):41–48.
21. Llovet JM, Di Bisceglie AM, Bruix J, et al. Design and endpoints of clinical trials in hepatocellular carcinoma. J Natl Cancer Inst 2008;100(10):698–711.
22. Talwalkar JA, Gores GJ. Diagnosis and staging of hepatocellular carcinoma. Gastroenterology 2004;127(5 Suppl 1):S126–S132.
23. Ferrara N. Vascular endothelial growth factor: molecular and biological aspects. Curr Top Microbiol Immunol 1999;237:1–30.
24. Burke AJ, Sullivan FJ, Giles FJ, Glynn SA. The yin and yang of nitric oxide in cancer progression. Carcinogenesis 2013;34(3):503–512.
25. Feng CW, Wang LD, Jiao LH, Liu B, Zheng S, Xie XJ.Expression of p53, inducible nitric oxide synthase and vascular endothelial growth factor in gastric precancerous and cancerous lesions: correlation with clinical features. BMC Cancer 2002;2:8.
26. Song ZJ, Gong P, Wu YE. Relationship between the expression of iNOS, VEGF, tumor angiogenesis and gastric cancer. World J Gastroenterol 2002;8(4):591–595.
27. Teerlink T, Luo Z, Palm F, Wilcox CS. Cellular ADMA: regulation and action. Pharmacol Res 2009;60(6):448–460.
28. Davids M, Teerlink T. Plasma concentrations of arginine and asymmetric dimethylarginine do not reflect their intracellular concentrations in peripheral blood mononuclear cells. Metabolism 2013;62(10):1455–1461.
29. Zhang P, Xu X, Hu X, et al. DDAH1 deficiency attenuates endothelial cell cycle progression and angiogenesis. PLoS ONE 2013;8(11):e79444.
30. Vanella L, Di Giacomo C, Acquaviva R, et al. The DDAH/NOS pathway in human prostatic cancer cell lines: antiangiogenic effect of L-NAME. J Oncol 2011;39(5):1303–1310.
31. Kostourou V, Robinson SP, Cartwright JE, Whitley GS. Dimethylarginine dimethylaminohydrolase I enhances tumour growth and angiogenesis. Br J Cancer 2002;87(6):673–680.
32. Zhu AX, Duda DG, Sahani DV, Jain RK. HCC and angiogenesis: possible targets and future directions. Nat Rev Clin Oncol 2011;8(5):292–301.
33. Singh S, Gupta AK. Nitric oxide: role in tumour biology and iNOS/NO-based anticancer therapies. Cancer Chemother Pharmacol 2011;76(6):1211–1224.

34. Wesseling P, Ruiter DJ, Burger PC. Angiogenesis in brain tumors; pathobiological and clinical aspects. J Neurooncol 1997;32(3):253–265.

35. Kostourou V, Cartwright JE, Johnstone AP, et al. The role of tumour-derived iNOS in tumour progression and angiogenesis. Br J Cancer 2011;104(1):83–90.

36. Cullis ER, Kalber TL, Ashton SE, et al. Tumour overexpression of inducible nitric oxide synthase (iNOS) increases angiogenesis and may modulate the anti-tumour effects of the vascular disrupting agent ZD6126. Microvasc Res 2006;71(2):76–84.

37. Lee JK, Abou-Alfa GK. An update on clinical trials in the treatment of advanced hepatocellular carcinoma. J Clin Gastroenterol 2013;47(Suppl):S16–S19.

38. Shen YC, Lin ZZ, Hsu CH, Hsu C, Shao YY, Cheng AL. Clinical trials in hepatocellular carcinoma: an update. Liver Cancer 2013; 2(3–4):345–364.

39. Chan SL, Yeo W. Targeted therapy of hepatocellular carcinoma: present and future. J Gastroenterol Hepatol 2012;27(5):862–872.

Discussion and future perspectives PART II

10

The results described in this thesis concerning the whole-body and interorgan pathway of glutamine, citrulline and arginine provide insights in the metabolic changes in the (cachexic) tumor-bearing host and provide new nutritional opportunities to counteract these disturbances.

In chapter six, a new hypothesized concept is demonstrated on the role of the glutamine, citrulline an arginine metabolism in development of cancer cachexia, explained and supported by an elaborate literature study. The arginine/NO metabolism is disturbed in patients with cancer. We theorized that the body will try to correct this perturbation by mobilizing arginine, citrulline and glutamine. The decreased arginine levels and the disturbed NO production activate several cascades, which in turn inhibit protein synthesis and promote proteolysis, leading to cachexia. In a recent study in mice, Marini et al. found no significant effect of arginine deprivation after administration of arginine deiminase on body weight and fractional protein synthesis rates (1). This chemical deprivation of arginine by arginine deiminase is accompanied by an increased citrulline level more than ten-fold, since arginine deiminase converts arginine into citrulline and ammonia. However, arginine deprivation caused by excessive arginase activity as seen in early phases of cancer development, converting arginine into ornithine and urea, results in severe weight loss and death, which can be counteracted by correction of citrulline levels (2). In conclusion, arginine deiminase does cause a depletion in circulating arginine, however this depletion is not systemic due to the ability of multiple cells to derive arginine from citrulline themselves. Catabolic syndromes are still a common problem in oncologic care and the presence of cancer related malnutrition may limit their recovery functions and responses to even the best treatments. This highlights the need for more scientific appraisal of metabolic derangements and nutritional requirements of cachectic subjects.

Metabolic flux studies, primarily in animals, have gained information on the qualitative interorgan uptake and release of the amino acids of interest. In chapter seven, the influence of the advanced tumor-bearing state with signs of cachexia on arginine's intestinal-renal axis is outlined. Our findings were in line with the mechanism described above. Previous studies in tumor-bearing rats have shown adaptations of arginine's intestinal-renal axis in different phases of malignant disease. In the first phase of cancer, arginine levels decrease without alterations in intestinal or renal metabolism. In a more advanced phase, the onset of cachexia is preceded by increases in glutamine release from muscle and citrulline production in the intestine, with a subsequent increase in renal arginine production (3-5). In

our tumor-bearing rats with advanced cancer cachexia, a high glutamine uptake by the small intestine was not attended by higher citrulline output and renal conversion of citrulline into arginine was not altered compared to non-tumor-bearing subjects. These observations suggest that citrulline is used as a substrate for metabolic and immunologic processes in times arginine metabolism is diminished. Our results imply that metabolic alterations found in tumor-bearing rats with advanced cancer cachexia do not depend on glutamine availability, but rather seem to be an adaptation process to tumor-induced changes in the host. Although a previous study showed that a glutamine enriched diet is able to restore host glutamine reserves and support glutamine metabolism, without stimulation of tumor growth (6), it seems that in advanced stages of cancer cachexia other nutritional interventions should be considered.

The arginine/NO pathway plays a crucial role in surgery, oncology and surgical oncology. In this thesis, it is shown that the arginine/NO pathway and the associated mediators are closely linked to angiogenetic pathways in primary liver cancer development. Furthermore, it was found that arginine supplementation in the perioperative setting improves long term (disease free) survival and recurrence of the malignant disease. The last outcome is of great importance, since it supports the hypothesis that arginine could prevent malignant outgrowth when available "in the right place at the right time".

Myeloid-derived suppressor cells (MDSC) are assigned to be major players in cancer's strategies for initiation, promotion and progression of malignant development and are a hallmark in cancer-associated inflammation discrepancies (7-10). In the multistep process of cancer development, these MDSC change their role and activity, depending on the phase of carcinogenesis. In the first phase, MDSC predominantly produce arginase induced by Th2 cytokines. This results in a high level of arginase activity, which causes an arginine deficiency. T-lymphocytes depend on arginine for proliferation and receptor production and therefore depleted arginine levels prevent these immune cells from fighting malignant cells (11,12). Also, increased NO concentrations, derived from arginine by iNOS, have been noticed to promote cytostasis and cytotoxicity in malignant cells and to induce expression the tumor suppressor gene p53 leading tot apoptosis (11,13-15). Evidently, in these early phases of cancer development, arginine is of great value to prevent malignant cells from evolving.

During further development of the malignant tumor, the progression phase of the carcinogenesis, the Th1/Th2 balance switches to an increase in Th1 cytokines,

promoting the MDSC to express iNOS and eNOS. This results in a continuous production of NO in the tumor environment, suppressing immune functions and promoting angiogenesis and tumor growth. Furthermore, together high iNOS activity and low arginine levels may lead to the formation of reactive nitrogen species, such as peroxynitrite. These highly reactive molecules damage the surrounding tissue promoting tumor outgrow and also cause immune suppression by blocking the binding of tumor-antigen to T-cell receptors. In this phase, extra arginine may cause harm by aggravating the pro-malignant effects of the MDSC in the tumor environment (16-18).

As described, bearing cancer results in a disturbance in the Th1/Th2 balance of the immune system. It is suggested that a curative surgical intervention for cancer may reverse the carcinogenetic pathway to the initiation phase with its Th2 response, as illustrated the phase where restoring arginine availability may have a protective role against tumor development. Furthermore, in general, the immune response has been found to shift to a domination of Th2 response and a decrease of the Th1 response after surgery (19). This shift further augments catabolism of arginine by arginase. This is supported by studies showing a decrease in arginine levels in patients just a few hours after surgical injury. Arginine supplementation may correct the Th1/Th2 imbalance in patients undergoing oncological surgery and hereby may enhance the innate and adaptive immune responses (20). This is supported by the fact that we found a better recurrence free survival in head and neck cancer patients receiving an arginine enriched enteral diet perioperatively.

Furthermore, tumor cells also change their own metabolic strategy to stimulate malignant outgrowth. In chapter eight, a more in-depth study on tumor cell metabolism and biology is presented. An important tumor growth promoting process is angiogenesis, in which new vasculature is formed to supply the tumor from oxygen-rich blood and nutritional compounds. From cardiovascular studies, we learned that the arginine/NO pathway is of major influence in the regulation of endothelial function, vascular condition and development. Significant angiogenesis requires adequate levels of NO. Various angiogenetic factors stimulate the expression of NOS isoenzymes. VEGF stimulates endothelial-derived NO and NO from the inducible enzyme stimulates VEGF expression in a positive feedback loop. The role of DDAH and ADMA in the regulation of NO biosynthesis in angiogenesis was also extensively investigated. DDAH overexpression results in the stimulation of the angiogenetic cascade (21). Overexpression of DDAH may augment a significant reduction in plasma ADMA levels and a significant

increase in tissue NOS activity with subsequent stimulating vascular effects (22). Furthermore, DDAH is able to augment complementary VEGF upregulation in a NO-independent manner (23). Together, increased DDAH activity lowers ADMA levels, increases the levels of VEGF, whilst blockade of DDAH reduces angiogenesis (21). Others demonstrated that downregulation of DDAH or treatment with ADMA impairs angiogenesis via a reduction in NO and VEGF signaling (21,25,26).

Functional studies looking into the DDAH/NO/VEGF pathway in the oncological setting show similar effects. Increased NO production has been associated with higher tumor grade in various types of malignancies, including gynaecological, breast, neuronal, head and neck and gastrointestinal tumors (27-31). Data from *in vitro* experiments showed that higher NO production resulted in tumor angiogenesis by upregulating VEGF and VEGF dependent neovascularisation (32,33). Conversely, inhibition of NOS results in decreased tumor growth (34). Moreover, suppression of iNOS in hepatocellular carcinoma cells led to decreased tumor growth and increased tumor cell death (35). Enhanced expression of DDAH increases NO formation, expression and secretion of VEGF, and hereby induced angiogenesis in malignant cells. Tumors with higher DDAH expression and thus higher NO levels grow more rapidly than tumors with normal DDAH expression and normal NO levels. Tumor cells that overexpress DDAH have a more aggressive and better-vascularized phenotype and the degree of DDAH expression can be correlated to the degree of hypoxia in the tumor environment (36-38). Thus, higher DDAH, iNOS, eNOS and VEGF expression is found in cancer cells compared to benign cells and inhibition of NOS shows a reduction in the overexpression of both NOS isoenzymes and VEGF-derived angiogenesis (39). In malignant cells overexpressing DDAH, a selective inhibitor of DDAH1 leads to downstream inhibition of NO biosynthesis (40). In conclusion, angiogenesis in cancer is stimulated by VEGF, VEGF augments NO formation to mediate angiogenesis, DDAH stimulates NO formation by catabolizing ADMA and NO and DDAH both also promote VEGF expression. The pathway of arginine, ADMA, DDAH, NOS and VEGF is summarized in a flow chart in Figure 1. We found that DDAH was upregulated in human primary liver cancer and that this increase in DDAH expression was accompanied by an increase in the arginine/ADMA ratio and subsequently enhanced NO formation and the angiogenesis promoting factors VEGF.

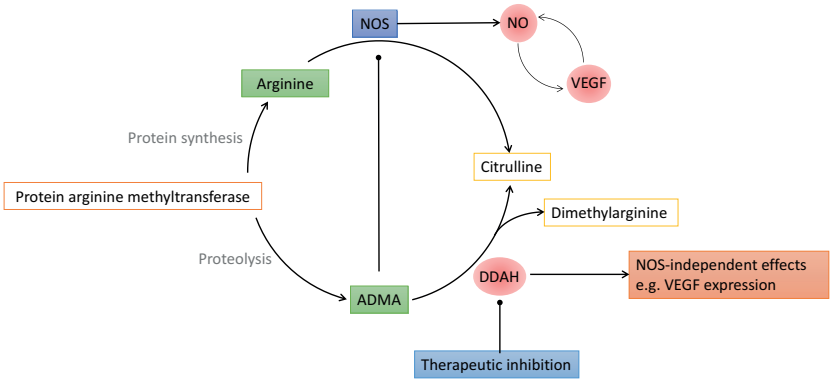

FIGURE 1 | The pathway of arginine, ADMA, DDAH, NOS and VEGF. Arginine is the sole precursor for NO via the three NOS isoforms. ADMA is the direct antagonist of arginine for NOS and thus inhibits NO production. DDAH converts ADMA into dimethylarginine and citrulline and hereby indirectly stimulates the formation of NO from arginine. VEGF directly stimulates the biosynthesis of NO. NO itself also augments VEGF expression in a positive feedback loop. DDAH also enhances VEGF dependent angiogenesis in a NOS independent manner.

Future persepectives

Influencing the arginine/NO pathway in oncological management is all about "in the right place at the right time". As described in the metabolic studies in this thesis, alterations in arginine metabolism may induce changes in protein turnover and immune function. Glutamine supplementation may be a useful intervention to restore citrulline and arginine levels in the first phases of cancer development. However, we also showed that in a more advanced phase with signs of cachexia, the body does not depend on glutamine administration anymore and seem to find other routes to warrant the arginine-derived processes by using citrulline as a surrogate instead. It would be of interest to study the role of the specific citrulline metabolism in the sequential phases of the carcinogenesis and the effect of nutritional interventions on the role of citrulline as arginine's surrogate in times of need.

Although multiple studies already have shown the role of arginine in tumor development and host immune function, the mechanism of how arginine supplementation may affect immune responses and metabolism on tissue or cell level still has to be unraveled. Arginine supplementation to patients in the

early phases of carcinogenesis or the perioperative phase could be beneficial in promoting the hosts immune system, however it would be even more interesting to tackle the real cause of the arginine deficiency state. Copious amounts of data show an association between MDSC accumulation and clinical outcome in patients with a malignancy (41,42). It was shown that MDSC are linked to poor disease specific and overall survival in patients with various tumor types (43). Furthermore, the level of MDSC is associated with lower responses to chemotherapies (44-46). This knowledge makes it particularly important to find new therapeutic options to target these cells. Various therapies, for example low dose chemotherapy, can induce MDSC depletion (47,48). MDSC can be functionally inactivated by unarming their suppressive mechanisms. For example, administration of tadalafil, a phosphodiesterase type 5 inhibitor, to patients with head and neck cancer and multiple myeloma resulted in lower circulating MDSC count, decreased iNOS and arginase expression and more spontaneously generated tumor-specific T-cells (49-51). Furthermore, interfering with the maturation pathway of the MDSC may lead to differentiation of the cells to another, non-pro-malignant phenotype.

Moreover, the perturbations in the arginine/NO metabolism may be counteracted by interventions that affect its mediators. Increased DDAH expression by cancer cells is an important key strategy in the stimulation of angiogenesis through induction of NO and VEGF formation, by inhibition of the antagonist function of ADMA. An interesting concept is to "inhibit the inhibitor". VEGF is a major target for anti-angiogenetic therapies, for example bevacizumab and sorafenib, showing promising therapeutic results in patients with different types of cancer. However, carcinogenesis and angiogenesis are multistep complex processes, not only involving VEGF. First, VEGF stimulates endothelial-derived NO and inducible NO stimulates VEGF expression. VEGF inhibitors have been shown to decrease NO levels, however this is only a downstream effect of the therapy (52). To optimize anti-VEGF therapy also the upstream stimulation by NO could be blocked. Second, NO does not only have tumor promoting effects through VEGF dependent angiogenesis, but NO itself is also important for tumor cell migration and progression (53). Thus, for optimal anti-tumorous effect, it would be essential to counteract upstream and downstream NO actions in the VEGF pathway in a horizontal and vertical way (54), and DDAH is a potential target to augment this additional contribution to the current available anti-VEGF therapies.

Direct inhibition of NO as a target in anti-cancer therapies will not only influence the pathological NO production, but may also disrupt physiological NO-mediated

processes as well. An alternative way to obviate these problems would be via indirect inhibition of NO synthesis by one of the DDAH isoforms. DDAH catabolizes ADMA, the most important inhibitor of NO synthesis because of its antagonist function for the NOS enzymes for arginine. ADMA is not a selective agent and will reduce both iNOS and eNOS production of NO. Therefore, the enzymatic action of DDAH is a potential endogenous mechanism for the regulation of NO formation by competitive inhibition (21). Furthermore, DDAH has been shown to also stimulate VEGF dependent angiogenesis in an NOS-independent manner.

Besides the known endogenous DDAH inhibitors, e.g. citrulline and homocysteine, very few commercially available inhibitors of DDAH are obtainable and most of these are not DDAH selective or lack DDAH isoform selectivity (54). Several of these compounds have been shown to inhibit both DDAH activity and NOS activity itself, hereby missing the point of the potential advantage of this target. Besides the problem of NOS inhibition by non-selective DDAH inhibitors, arginase inhibition is described as well. This could result in higher arginine levels, the precursor for NO synthesis via NOS, controversially leading to higher NO production. Therefore, the optimal pharmalogical agent that reduces NO formation via DDAH inhibition does not inhibit NOS itself or arginase. Currently, only two of these synthetic DDAH inhibitors have been described showing selectivity for DDAH over NOS and arginase, namely the guanidine substituted arginines NG-(2-methoxyethyl)-L-arginine(L-257) and NG-(2-methoxyethyl)-L-arginine methylester(L-291) (55,56).

Selective inhibition of one of the DDAH isoforms could result in a tissue-specific rise of ADMA and subsequently inhibition of NOS. This may also prevent systemic NOS blockade, seen in direct NOS inhibition by administration of methylated arginines and anti-VEGF, leading to serious cardiovascular adaptations associated with diminished clinical outcome in septic patients (57,58). Another, even more innovative, option to prevent systemic effects of VEGF and DDAH inhibitors, is the use of microbubbles to deliver the therapeutic agents at the tumor site. First, this new method enables tissue specific target localization by microbubbles conjugated with a biomarker, specific for the tissue of interest. Second, the microbubbles can be loaded with a therapeutic agent, which can be released at the targeted tumor site by the use of ultrasound. In summary, there are numerous new and exciting potential options of DDAH inhibition alone and in combination with other anti-angiogenetic therapies.

References

1. Marini JC, Didelija IC. Arginine depletion by arginine deiminase does not affect whole protein metabolism or muscle fractional protein synthesis rate in mice. PLoS One. 2015 Mar 16;10(3):e0119801.
2. Mauldin JP, Zeinali I, Kleypas K et al. Recombinant human arginase toxicity in mice is reduced by citrulline supplementation. Transl Oncol 2012;5(1):26-31.
3. de Blaauw I, Heeneman S, Deutz NE, Von Meyenfeldt MF. Increased whole-body protein and glutamine turnover in advanced cancer is not matched by an increased muscle protein and glutamine turnover. J Surg Res 1997;68(1):44-55.
4. de Blaauw, I, Deutz NE, van der Hulst RR, von Meyenfeldt MF. Glutamine depletion and increased gut permeability in nonanorectic, non-weight-losing tumor-bearing rats. Gastroenterology 1997;112(1):118-126.
5. Souba WW, Strebel FR, Bull JM, Copeland EM, Teagtmeyer H, Cleary K. Interorgan glutamine metabolism in the tumor-bearing rat. J Surg Res 1988;44(6):720-726.
6. Klimberg VS, Souba WW, Sailoum RM, et al. Glutamine-enriched diets support muscle glutamine metabolism without stimulating tumor growth. J Surg Res 1990;48(4):319-323.
7. Bronte V, Serafini P, Mazzoni A, Segal DM, ZanovelloP. L-arginine metabolism in myeloid cells controls T-lymphocyte functions. Trends Immunol 2003;24(6):302-6.
8. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol 2009;9(3):162-174.
9. Marx J. Cancer immunology. Cancer's bulwark against immune attack: MDS cells. Science 2008;319(5860):154-156.
10. Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. J Immunol 2009;182(8):4499-4506.
11. Redente EF, Orlicky DJ, Bouchard RJ, Malkinson AM. Tumor signaling to the bone marrow changes the phenotype of monocytes and pulmonary macrophages during urethane-induced primary lung tumorigenesis in A/J mice. Am J Pathol 2007 Feb;170(2):693-708.
12. Rodriguez PC, Quiceno DG, Zabaleta J. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. Cancer Res. 2004 Aug 15;64(16):5839-49.
13. Bogdan C. Nitric oxide synthase in innate and adaptive immunity: an update. Trends Immunol 2015;36(3):161-178.
14. Messmer UK, Ankarcrona M, Nicotera P, Brüne B. p53 expression in nitric oxide-induced apoptosis. FEBS Lett 1994;355(1):23-26
15. Nicotera P, Bonfoco E, Brüne B. Mechanisms for nitric oxide-induced cell death: involvement of apoptosis. Adv Neuroimmunol 1995;5(4):411-420.
16. Burke AJ, Sullivan FJ, Giles FJ, Glynn SA. The Yin and Yang of Nitric Oxide in Cancer Progression. Carcinogenesis 2013;34(3):503-12.
17. Ma Q, Wang Z, Zhang M, et al. Targeting the L-arginine-nitric oxide pathway for cancer treatment. Curr Pharm Des. 2010;16(4):392-410.
18. Raber PL, Thevenot P, Sierra R et al. Subpopulations of myeloid-derived suppressor cells impair T cell responses through independent nitric oxide-related pathways. Int J Cancer 2014;134(12):2853-2864.

19. Marik PE, Flemmer M. The immune response to surgery and trauma: Implications for treatment. J Trauma Acute Care Surg 2012;73(4):801-808.
20. Matsuda A, Furukawa K, Takasaki H, et al. Preoperative oral immune-enhancing nutritional supplementation corrects TH1/TH2 imbalance in patients undergoing elective surgery for colorectal cancer. Dis Colon Rectum 2006;49(4):507-516.
21. Fiedler LR, Bachetti T, Leiper J, et al. The ADMA/DDAH pathway regulates VEGF mediated angiogenesis. Arterioscler Thromb Vasc Biol 2009;29:2117-2124
22. Dayoub H, Acham V, Adimoolam S, et al. Dimethylarginine dimethylaminohydrolase regulates nitric oxide synthesis: genetic and physiological evidence. Circulation 2003;108:3042–3047.
23. Hasegawa K, Wakino S, Tanaka T, et al. Dimethylarginine dimethylaminohydrolase 2 increases vascular endothelial growth factor expression through Sp1 transcription factor in endothelial cells. Arterioscler Thromb Vasc Biol 2006;26:1488–1494.
24. Smith CL, Birdsy GM, Anthony S, Arrigoni FI, Leiper JM, Vallance P. Dimethylarginine dimethylaminohydrolase activity modulates ADMA levels, VEGF expression, and cell phenotype. Biochem Biophys Res Commun 2003;308:984-989.
25. Wojciak-Stothard B, Torondel B, Tsang LY, et al. The ADMA/DDAH pathway is a critical regulator of endothelial cell motility. J Cell Sci. 2007;120:929–942.
26. Dowsett L, Piper S, Slaviero A, et al. Endothelial Dimethylarginine Dimethylaminohydrolase 1 Is an Important Regulator of Angiogenesis but Does Not Regulate Vascular Reactivity or Hemodynamic Homeostasis. Circulation. 2015 Jun 23;131(25):2217-25.
27. Thomsen LL, Miles DW, Happerfield L, Bobrow LG, Knowles RG, Moncada S. Nitric oxide synthase activity in human breast cancer. Br. J. Cancer 1995;72:41–44.
28. Cobbs CS, Brenman JE, Aldape KD, Bredt DS, Israel MA. Expression of nitric oxide synthase in human central nervous system tumors. Cancer Res 1995;55:727–730.
29. Tanriover N, Ulu MO, Isler C, et al. Neuronal nitric oxide synthase expression in glial tumors: correlation with malignancy and tumor proliferation. Neurol Res 2008;30:940–944.
30. Ng QS, Goh V, Milner J, et al. Effect of nitric-oxide synthesis on tumour blood volume and vascular activity: a phase I study. Lancet Oncol 2007;8:111–118.
31. Tse GM, Wong FC, Tsang AK, et al. Stromal nitric oxide synthase (NOS) expression correlates with the grade of mammary phyllodes tumour. J Clin Pathol 2005;58:600–604.
32. Nakamura Y, Yasuoka H, Tsujimoto M, et al. Nitric oxide in breast cancer: induction of vascular endothelial growth factor-C and correlation with metastasis and poor prognosis. Clin Cancer Res 2006;12:1201–1207.
33. Nakamura Y, Yasuoka H, Zuo H, et al. Nitric oxide in papillary thyroid carcinoma: induction of vascular endothelial growth factor D and correlation with lymph node metastasis. J Clin Endocrinol Metab 2006;91:1582–1585.
34. Jadeski LC, Hum KO, Chakraborty C, Lala PK. Nitric oxide promotes murine mammary tumour growth and metastasis by stimulating tumour cell migration, invasiveness and angiogenesis. Int J Cancer 2000;86:30-39.
35. Calvisi DF, Pinna F, Ladu S, et al. Aberrant iNOS signaling is under genetic control in rodent liver cancer and potentially prognostic for the human disease. Carcinogenesis 2008;29:1639–1647.

36. Kostourou V, Robinson SP, Cartwright JE, Whitley GS. Dimethylarginine dimethylaminohydrolase I enhances tumour growth and angiogenesis. Br J Cancer 2002;87:673–680.

37. Kostourou V, Robinson SP, Cartwright JE, Griffiths JR. Effects of overexpression of dimethylarginine dimethylaminohydrolase on tumor angiogenesis assessed by susceptibility magnetic resonance imaging. Cancer Res 2003;63;4960–4966.

38. Kostourou V, Troy H, Murray JF, et al. Overexpression of dimethylarginine dimethylaminohydrolase enhances tumor hypoxia: an insight into the relationship of hypoxia and angiogenesis *in vivo*. Neoplasia 2004; 6:401-411.

39. Vanella L, Di Giacomo C, Acquiviva R, et al. The DDAH/NOS pathway in human prostatic cancer cell lines: antiangiogenic effect of L-NAME. Int J Oncol 2011;39:1303-1310.

40. Wang Y, Hu S, Gabisi AM jr, et al. Developing an irreversible inhibitor of human DDAH-1, an enzyme upregulated in melanoma. Chem Med Chem 2014;9:792-797.

41. Messmer MN, Netherby CS, Banik D, Abrams SI. Tumor-induced myeloid dysfunction and its implications for cancer immunotherapy. Cancer Immunol Immunother 2015;64:1–13.

42. Olito S, Marigo I, Pinton L, Damuzzo V, Mandruzzato S, Bronte V . Myeloid-derived suppressor cell heterogeneity in human cancers. Ann NY Acad Sci 2014;1319:47–65.

43. Zhang S, Ma X, Zhu C, Liu L, Wang G, Yuan X. The role of myeloid-derived suppressor cells in patients with solid tumors: A meta-analysis. PLoS One 2016;11:e0164514.

44. Finke J, Ko J, Rini B, Rayman P, Ireland J, Cohen P. MDSC as a mechanism of tumor escape from sunitinib mediated anti-angiogenic therapy. Int Immunopharmacol 2011;11:856–61.

45. Ramachandran IR, Condamine T, Lin C, et al. Bone marrow PMN-MDSCs and neutrophils are functionally similar in protection of multiple myeloma from chemotherapy. Cancer Lett 2016;371:117–124.

46. Meyer C, Cagnon L, Costa-Nunes CM, et al.Frequencies of circulating MDSC correlate with clinical outcome of melanoma patients treated with ipilimumab. Cancer Immunol Immunother 2014;63:247–257.

47. Suzuki E, Kapoor V, Jassar AS, Kaiser LR, Albelda SM. Gemcitabine selectively eliminates splenic Gr-1+/CD11b+ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity. Clin Cancer Res 2005;11:6713–6721.

48. Vincent J, Mignot G, Chalmin F, et al. 5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity. Cancer Res 2010;70:3052–3061.

49. Califano JA, Khan Z, Noonan KA, et al. Tadalafil augments tumor specific immunity in patients with head and neck squamous cell carcinoma. Clin Cancer Res 2015;21:30–38.

50. Weed DT, Vella JL, Reis IM, et al. Tadalafil reduces myeloid-derived suppressor cells and regulatory T cells and promotes tumor immunity in patients with head and neck squamous cell carcinoma. Clin Cancer Res 2015;21:39–48.

51. Noonan KA, Ghosh N, Rudraraju L, Bui M, Borrello I. Targeting immune suppression with PDE5 inhibition in end-stage multiple myeloma. Cancer Immunol Res 2014;2:725–31

52. Shen BQ Lee DY, Zioncheck TF. Vascular endothelial growth factor governs endothelial nitric-oxide synthase expression via a KDR/Flk-1 receptor and a protein kinase C signalling pathway. J Biol Chem 1999;274:33057–33063.

53. Ma Q, Wang Z, Zhang M, et al. Targeting the L-arginine-nitric oxide patway for cancer treatment. Curr Pharm Des 2010;16:392-410.

54. Leiper J, Nandi M. The therapeutic potential of targeting endogenous inhibitors of nitric oxide synthesis. Nat Rev Drug Discov 2011;10:277-291.

55. Rossiter S, Smith CL, Malaki M, et al. Selective substrate-based inhibitors of mammalian dimethylarginine dimethylaminohydrolase. J Med Chem 2005:48:4670–4678.

56. Wang Z, Lambden S, Taylor V, et al. Pharmacological inhibition of ddah1 improves survival, haemodynamics and organ function in experimental septic shock. Biochem J 2014;460:309–316.

57. Lopez A, Lorente JA, Steingrub J, et al. Multiple-center, randomized, placebo-controlled, double-blind study of the nitric oxide synthase inhibitor 546C88: effect on survival in patients with septic shock. Crit. Care Med 2004;32:21–30.

58. Leiper J, Nandi L, Torondel B, et al. Disruption of methylarginine metabolism impairs vascular homeostasis. Nature Med 2007;13:198–203.

Summary / Samenvatting
List of Publications
Curriculum Vitae
Aknowledgements

11

Summary

The objective of this thesis was to gain insight in the role of the arginine/NO metabolism in surgical oncology, from molecular level to whole-body level. In PART I background information on the arginine and its derivatives is provided and the physiological arginine/NO pathway is further elucidated. Arginine and its precursors have been shown to be essential pharmaco-nutrients due to their immune enhancing capacities (CHAPTER TWO). An adequate immune response is particularly important in patients undergoing surgery for recovery from injury and combating various diseases. A surgical intervention causes stress with subsequent catabolic effects on the body's substrate stores. Glutamine, the precursor of arginine, functions as fuel for rapidly dividing cells, especially cells of the immune system. Also, it activates protective mechanisms of the host because it is an important precursor for antioxidants and it improves intestinal function. Moreover, arginine is the substrate for T-lymphocytes and the sole precursor for NO production, both essential for the immune system. Supplementation of these pharmaco-nutrients in surgical patients may improve clinical outcome, since these nutrients become deficient fast under the influence of surgical stress and in disease states.

Glutamine is the precursor for arginine and glutamine supplementation augments an increase in arginine levels in healthy subjects. We designed a stable isotope study to determine the effect of intravenous glutamine supplementation on arginine production from glutamine and citrulline (CHAPTER THREE AND FOUR). It was found that an intravenous glutamine supplement in patients undergoing abdominal surgery doubles renal arginine production.

PART II of this thesis focuses on arginine/NO metabolism in surgical oncology. The nutritional status of patients is a major prognostic factor. Especially cancer patients may develop a severe catabolic state, also called cancer cachexia (CHAPTER SIX). The presence of cachectic and sarcopenic symptoms is significantly related to survival in oncologic patients. It was hypothesized that part of the pathological changes in cancer cachexia can be ascribed to an arginine deficiency state, leading to excessive changes in amino acid metabolism. It is shown in previous studies that the tumor-bearing host accelerates arginine's intestinal-renal axis by glutamine mobilization from skeletal muscle and this may promote cachexia. In CHAPTER SEVEN we describe a metabolic flux study in rats with advanced cancer cachexia. Amino acid fluxes and net fractional extractions across intestine,

kidneys, and liver were studied. In the advanced cachectic tumor-bearing state, an increase in intestinal glutamine uptake is not accompanied by an increase in renal arginine production. The adaptations found in the cachectic, tumor-bearing rat did not depend on glutamine availability.

An arginine deficiency state is typically found in tumor-bearing patients, and this diminishes immune responses against malignant degeneration. In CHAPTER EIGHT a randomized trial on the clinical effect of arginine supplementation in surgical oncology is outlined. In this double-blind trial, we randomly assigned severely malnourished patients with head and neck cancer to receive a standard perioperative enteral nutrition or an arginine-supplemented perioperative enteral nutrition. We did not observe significant differences in baseline characteristics. The group receiving an arginine-enriched diet had a significantly better overall survival and a significantly better locoregional recurrence-free survival. This supports the thought that arginine availability is particular important at the moment one requires the most of the immune system for recovery from surgical injury and to encounter remnant malignant cells in the perioperative period.

The function of both arginine and NO are elaborately studied in the tumor environment. However, the role of other mediators in the arginine/NO pathway in surgical oncology is poorly represented in literature. NO production depends on arginine as substrate and asymmetric dimethylarginine (ADMA) as inhibitor. Dimethylarginine dimethylaminohydrolase (DDAH) catabolizes ADMA, and hereby regulates NO production. Solid tumors need vasculature to evolve and therefore angiogenesis is an essential part of cancer development. Vascular endothelial growth factor (VEGF) is a crucial component in cancer angiogenesis and uses NO as a mediator. To study the role of ADMA and DDAH in the angiogenetic pathway of primary liver cancer, we analyzed the resection specimens of twenty patients (CHAPTER NINE). Our results indicate that DDAH expression is increased in human hepatocellular carcinoma, which is associated with an increase of the arginine/ADMA ratio and enhanced NO formation. The increased DDAH expression is initiated by hypoxia and is associated with promotion of the expression of the angiogenesis stimulating factor VEGF. This suggests that DDAH might be a potential target in novel therapeutic agents and future studies are warranted to investigate the role of the arginine/NO pathway with all its mediators in cancer development and anti-cancer strategies.

Samenvatting

Het doel van dit proefschrift is om inzicht te verkrijgen in de rol van het arginine/NO metabolisme in de chirurgische oncologie, van moleculair- tot lichaamsniveau. In DEEL I geven we achtergrondinformatie over arginine en haar derivaten en proberen we de fysiologische pathway van arginine en NO te verhelderen.

Arginine en haar voorlopers zijn essentiële farmaconutriënten vanwege hun immuunmodulerende capaciteiten (HOOFDSTUK TWEE). Een adequate immuunrespons is essentieel in patiënten die herstellen van een operatie of lijden aan een ernstige ziekte. Een chirurgische interventie veroorzaakt stress, resulterend in katabolisme van de substraatvoorraden van het lichaam. Glutamine, de voorloper van arginine, functioneert als brandstof voor snel delende cellen, in het bijzonder cellen van het immuunsysteem. Daarnaast activeert glutamine belangrijke beschermende mechanismen in het lichaam, zo is het de voorloper van antioxidanten en behoudt het de gastro-intestinale functie. Arginine is het substraat voor T-lymfocyten en de unieke voorloper voor NO productie, beide essentieel voor een goed functionerend immuunsysteem. Omdat deze aminozuren snel deficiënt raken onder invloed van chirurgische stress en uiteenlopende ziekten, kan suppletie van deze farmaconutriënten de klinische uitkomst optimaliseren.

Glutamine is de precursor voor arginine en glutamine suppletie resulteert in een verhoging van de argininеconcentraties in gezonde proefpersonen. We verrichtten een onderzoek met behulp van stabiele isotopen om het effect van intraveneuze glutamine suppletie op de arginine productie van glutamine en citrulline te bestuderen (HOOFDSTUK DRIE EN VIER). Het bleek dat intraveneuze toediening van glutamine aan patiënten die een abdominale operatie ondergaan leidt tot een verdubbeling in de productie van renale arginine.

In DEEL II van het proefschrift wordt de focus gelegd op het arginine/NO metabolisme in de oncologische chirurgie. De voedingsstatus is een belangrijke prognostische factor voor een verscheidenheid aan ziektebeelden en behandelingen. Vooral kankerpatiënten kunnen een katabole status ontwikkelen, ook kanker-cachexie genoemd (HOOFDSTUK ZES). De aanwezigheid van cachectische en sarcopene symptomen is significant gerelateerd aan overleving bij oncologische patiënten. Een deel van de pathologische veranderingen in kanker cachexie zou kunnen worden toegeschreven aan kanker gerelateerde argininedeficiëntie en uitgebreide veranderingen in het aminozuurmetabolisme

van de gastheer. Er werd eerder al aangetoond dat de aanwezigheid van een maligniteit leidt tot een acceleratie van de intestinalel-renale as door toegenomen glutaminemobilisatie uit skeletspieren en dit zou het ontstaan van cachexie kunnen ontketenen. In HOOFDSTUK ZEVEN beschrijven we een metabool flux onderzoek in ratten met gevorderde kanker cachexie. Aminozuurfluxen en fractionele extracties over darm, nieren en lever werden bestudeerd. In de ratten met gevorderde kanker cachexie zagen we een verhoging van de opname van glutamine in de darm, echter ging dit niet gepaard met een toename van de productie van arginine door de nieren. Deze metabole veranderingen in de cachectische, tumor-dragende ratten waren niet afhankelijk van de glutamine beschikbaarheid.

In patiënten met kanker wordt frequent een arginine-deficiëntie gevonden en dit zou de immuunrespons tegen maligne ontaarding kunnen beperken. In HOOFDSTUK ACHT wordt een studie naar het klinische effect van argininesuppletie in de chirurgische oncologie beschreven. In dit dubbelblinde gerandomiseerde onderzoek kregen ernstig ondervoede patiënten met hoofd-halskanker een standaard perioperatieve enterale voeding of een met arginine verrijkte perioperatieve enterale voeding. Er werden geen significante verschillen waargenomen in de basiskarakteristieken. De groep die een met arginine verrijkte voeding kreeg, had een significant betere totale overleving en een significant betere ziekte-vrije overleving. Dit ondersteunt onze hypothese dat adequate arginine levels essentieel zijn voor het immuunsysteem tijdens het herstel van chirurgisch letsel en ter bestrijding van eventueel achtergebleven kwaadaardige cellen in de perioperatieve periode.

De functie van zowel arginine als NO is uitvoerig bestudeerd in de oncologie. De rol van andere mediatoren van de arginine/NO pathway in de oncologische setting wordt nog nauwelijks beschreven in de literatuur. NO synthese is afhankelijk van arginine als substraat en asymmetrische dimethylarginine (ADMA) als remmer van NO synthase. Dimethylarginine dimethylaminohydrolase (DDAH) katalyseert de afbraak van ADMA en reguleert daardoor de productie van NO. Solide tumoren hebben vascularisatie nodig om te groeien en daarom is angiogenese een essentieel onderdeel van de ontwikkeling van kanker. Vascular endothelial growth factor (VEGF) is een cruciale component in het proces van angiogenese en NO is zijn belangrijkste mediator. Om de rol van ADMA en DDAH in angiogenese van primaire leverkanker te bestuderen, analyseerden we de resectiepreparaten van twintig patiënten (HOOFDSTUK NEGEN). Onze resultaten toonden dat de

DDAH-expressie verhoogd is in humaan hepatocellulair carcinoom, wat gepaard gaat met een toename van de arginine/ADMA-verhouding en toegenomen NO synthese. De verhoogde DDAH expressie wordt geïnitieerd door hypoxie en is geassocieerd met een verhoogde expressie van de angiogenese stimulerende factor VEGF. Dit suggereert ook dat DDAH mogelijk een potentieel target is in nieuwe therapeutische strategieën. Toekomstige studies zijn nodig om de rol van de arginine/NO pathway met al zijn spelers in de ontwikkeling van kanker en in potentiele anti-kankerstrategieën te verhelderen.

Publications

1. Buijs N, Oosterink JE, Jessup M, Schierbeek H, Stolz DB, Houdijk AP, Geller DA, van Leeuwen PA. A new key player in VEGF-dependent angiogenesis in human hepatocellular carcinoma: dimethylarginine dimethylaminohydrolase 1. Angiogenesis 2017; 20(4): 557-565.

2. Buijs N, Brinkmann SJH, Ligthart-Melis GC, Schierbeek H. Chapter 6: Metabolism of glutamine, citrulline and arginine; stable isotopes analyzing the intestinal-renal axis. In: Schierbeek H, eds. Mass spectrometry and stable isotopes in nutritional and pediatric research. 1st ed. Hoboken, New Jersey: John Wiley & Sons, Inc., 2017: 139-167.

3. Vermeulen MA, Brinkmann SJ, Buijs N, Beishuizen A, Bet PM, Houdijk AP, van Goudoever JB, van Leeuwen PA. Enteral Glutamine Administration in Critically Ill Nonseptic Patients Does Not Trigger Arginine Synthesis. Journal of Nutrition and Metabolism 2016; 2016: 1373060.

4. Buijs N, Brinkmann SJ, Vermeulen MA, Oosterink E, Schierbeek H, Beishuizen A, de Vries JP, Wisselink W, van Leeuwen PA. Perioperative glutamine supplementation restores disturbed renal arginine synthesis after open aortic surgery: a randomized controlled clinical trial. American Journal of Physiology. Renal Physiology 2016; 311(3): F567-75.

5. Luttikhold J, van Norren K, Rijna H, Buijs N, Ankersmit M, Heijboer AC, Gootjes J, Hartmann B, Holst JJ, van Loon LJ, van Leeuwen PA. Jejunal feeding is followed by a greater rise in plasma cholecystokinin, peptide YY, glucagon-like peptide 1, and glucagon-like peptide 2 concentrations compared with gastric feeding *in vivo* in humans: a randomized trial. American Journal of Clinical Nutrition. 2016; 103(2): 435-43.

6. Luttikhold J, van Norren K, Buijs N, Ankersmit A, Heijboer AC, Gootjes J, Rijna H, van Leeuwen PAM, van Loon LJC. Jejunal casein feeding is followed by more rapid protein digestion and amino acid absorption when compared with gastric feeding in healthy young man. Journal of Nutrition 2015; 145(9): 2033-8.

7. Brinkmann SJH, Wörner, Buijs N, Richir M, van Leeuwen PAM, Cynober L. The arginine/ADMA ratio is related to the prevention of atherosclerotic plaques in hypercholesterolemic rabbits when giving a combined therapy with atorvastatine and L-arginine. International Journal of Molecular Sciences 2015; 16(6): 12230-42.

8. Buijs N, Weeda V, Vermeulen MAR, Bading J, Houdijk APJ, van Leeuwen PAM. Arginine's intestinal-renal axis is disturbed in the cachectic tumor-bearing rat. Nutrition and Cancer 2015; 67(5): 713-20.

9. Buijs N, Brinkmann SJ, Oosterink JE, Luttikhold J, Schierbeek H, Wisselink W, Beishuizen A, van Goudoever JB, Houdijk AP, van Leeuwen PA, Vermeulen MA. Reply to GC Ligthart-Melis et al. American Journal of Clinical Nutrition 2015; 101(4): 892-3.

10. Buijs N, Brinkmann SJH, Oosterink JE, Luttikhold J, Schierbeek H, Wisselink W, Beishuizen A, van Goudoever JB, Houdijk APJ, van Leeuwen PAM, Vermeulen MAR. Intravenous glutamine supplementation enhances renal *de novo* arginine synthesis in humans; a stable isotope study. American Journal of Clinical Nutrition 2014; 100: 1385-91.

11. Buijs N, van Leeuwen PAM. Standard vs. Enriched high protein enteral nutrition in the ICU. JAMA 2014; 312(21): 2288-9.

12. Oosterink JE, Buijs N, van Goudoever JB, Schierbeek H. A novel method for simultaneous measurement of concentration and enrichment of NO synthesis-specific amino acids in human plasma using stable isotopes and LC/MS ion trap analysis. Journal of Chromatography B. Analytical Technologies in the Biomedical and Life Sciences 2014; 958: 10-5.

13. Luttikhold J, van Norren K, Minor M, Buijs N, van den Braak CCM, Ludwig T, Abrahamse E, Rijna H, van Leeuwen PAM. The effect of fibers on coagulation of casein-based enteral nutrition in an artificial gastric digestion model. Food & Function 2014; 5(8): 1866-71.

14. Brinkmann SJ, de Boer MC, Buijs N, van Leeuwen PA. Asymmetric dimethylarginine and critical illness. Current Opinion in Clinical Nutrition and Metabolic Care 2014; 17(1): 90-7.

15. Buijs N, Vermeulen MAR, van Leeuwen PAM. Glutamine and Antioxidants in Critically Ill Patients. New England Journal of Medicine 2013; 369(5): 484.

16. Buijs N, Wörner E, Luttikhold J, Brinkmann SJH, Houdijk APJ, van Leeuwen PAM. Novel nutritional substrates in Surgery. The Proceedings of the Nutrition Society 2013; 15: 1-11.

17. Luttikhold J, Buijs N, van Norren K, Rijna H, van Leeuwen PAM. Gastrointestinal obstruction by solidification of enteral nutrition: a result of impaired digestion in critically ill. Nutritional Therapy & Metabolism 2013; 31(3): 101-9.

18. Brinkmann SJH, Buijs N, Mahdavian Delavary B, Luttikhold J, Niessen FB, van Leeuwen PAM. Preoperative oral nutritional interventions in surgery, including arginine and glutamine-enhanced supplements. CAB Reviews 2013; 8:1-14.

19. Buijs N, Marges ER, Visser M, Van Leeuwen PAM. Capítulo 65: Arginina en Nutrición especializada. In: Anaya Prado R, Arenas Marquéz H, Arenas Moya D, eds. Nutrición enteral y parenteral. 2 ed. Santa Fé: McGraw-Hill Interamericana, 2012: 584-595.

20. Buijs N, Luttikhold J, Houdijk APJ, Van Leeuwen PAM. The role of a disturbed arginine/NO metabolism in the onset of cancer cachexia: a working hypothesis. Current Medicinal Chemistry 2012;19(31):5278-86.

21. Buijs N, Van Bokhorst-de van der Schueren MAE, Langius JAE, Leemans CR, Kuik DJ, Vermeulen MAR, Van Leeuwen PAM. Perioperative arginine-supplemented nutrition in malnourished patients with head and neck cancer improves long-term survival. American Journal of Clinical Nutrition 2010; 92: 1151-6

Awards & Grants

- Travel Grant Food to fight cancer. Stichting Vivax, Amsterdam, 2013.
- Research Grant. Arginine/NO metabolism in primary liver cancer. Research project UPMC, Pittsburgh, USA. Egbers Stichting, Rotterdam, 2012.
- AstraZeneca Student Award. NVGE voorjaarsvergadering, Veldhoven, 2008.

Curriculum vitae

Nikki Buijs was born on the ninth of April 1986 in Amersfoort, the Netherlands. With physical therapists as parents, her affection for health care started early. In 2004, she graduated from secondary school, Stedelijk Gymnasium Johan van Oldebarnevelt in Amersfoort, and got accepted to VU University Medical School. During her studies, Nikki was particularly interested in surgery and in 2007 she contacted prof. dr. P.A.M. Van Leeuwen for an elective in experimental surgery. He introduced her to scientific research and in 2008 she won an award from the Dutch Society of Gastroenterology for her work. Before obtaining her medical degree in 2011 she attended a surgical training program for clinical officers in rural hospitals in Malawi and published her first scientific manuscript.

Prof. dr. P.A.M. Van Leeuwen got her motivated to start a PhD project on nutrition and metabolism in surgical oncology under his and dr. A.P.J Houdijk's supervision. She worked full time as a researcher at the department of surgery of the VU University Medical Center for approximately two and a half years. For one of the study protocols she left Amsterdam for Pittsburgh, where she worked as a research fellow at the Liver Cancer Center of the University of Pittsburgh Medical Center under supervision of prof. dr. D.A. Geller.

Nikki obtained clinical experience as a resident not in training at the general surgery department of Medical Center Alkmaar under guidance of dr. W.H. Schreurs. In 2015, she started her postgraduate training in general surgery in Zaandam Medical Center with dr. F.C. Den Boer as her mentor. During this time, she also got the opportunity to participate in a surgical training project of the Morogoro Support Foundation in St. Kizito hospital in Mikumi, Tanzania. In July 2018, she will return to the department of surgery of VU University Medical Center for the academic part of her surgical residency, with prof. dr. D.L. van der Peet as regional program director.

Acknowledgements

Numerous people have contributed to this thesis. My endless gratitude to you all.

Special thanks go out to:

My promotor and mentor in work and life, prof. dr. P.A.M. van Leeuwen
My copromotor, dr. A.P.J. Houdijk
My paranymphs and dear friends, Joanna Luttikhold and Femke Stip
The best research colleagues I could ever imagine
My PhD sisters
All my colleagues at the Department of Surgery at VU University Medical Center
My colleagues at the Department of Intensive Care, Anesthesiology, Dietetics, Pharmacology and Clinical Chemistry at VU University Medical Center
My respected colleague dr. H. Schierbeek and his Mass Spectrometry team at Academic Medical Center Amsterdam
My colleagues and new friends from Pittsburgh
My colleagues at the Department of Vascular Surgery, Pharmacology and Clinical Chemistry at St. Antonius Hospital Nieuwegein
All my co-authors
All the participating patients

My colleagues at the Department of Surgery at Medical Center Alkmaar
My colleagues at the Department of Surgery at Zaans Medical Center

My parents, for their infinite support and enthusiasm
My favorite person in the world